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This is a request for filing a (X) continuation application under 37 CFR 1.53(b), of pending prior application Serial No. **08/864,955** filed on **May 29, 1997**, of:

David Beach and Konstantin Galaktionov; Entitled: Novel cdc25 Genes, Encoded Products and Uses Thereof

Enclosed are:

96 page(s) of specification
4   page(s) of claims
1   page(s) of abstract
25 sheet(s) of drawing
5   page(s) of executed declaration and power of attorney

	CLAIMS	NO. FILED	NO. EXTRA	RATE	CALCULATIONS
	TOTAL CLAIMS (37 CFR 1.16(c))	-20=		x $22.00=	$
	INDEPENDENT CLAIMS (37 CFR 1.16(b))	-3=		x $82.00=	
	MULTIPLE DEPENDENT CLAIMS (if applicable) (37 CFR 1.16(d))			+ $270.00=	
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			Total of above Calculations =		
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October 30, 2000
Date

James T. Olesen Ph.D.
Registration No. 46, 967
Agent for Applicants

20/429217.1

Novel Human cdc25 Genes, Encoded Products and Uses Thereof

<u>Related Applications</u>

This application is a continuation-in-part of U.S.S.N.
08/379,685 filed 26 January 1995, which is a continuation-in-part
of U.S.S.N. 08/124,569, filed 20 September 1993, which is a
continuation-in-part of U.S.S.N. 07/793,601, filed 18 November
1991, and is a continuation-in-part of U.S.S.N. 08/189,206, filed
31 January 1994, which is a continuation-in-part of U.S.S.N.
07/878,640, filed 5 May 1992, and is a continuation-in-part of
U.S.S.N. 07/793,601, filed 18 November 1991, the specification
and claims of which are incorporated by reference herein.

<u>Background of the Invention</u>

In eukaryotic cells, mitosis is initiated following
the activation of a protein kinase known as "M-phase
promoting factor" (MPF; also known as the H-phase specific
histone kinase, or more simply as the H-phase kinase).
This kinase consists of at least three subunits: the
catalytic subunit (cdc2), a regulatory subunit (cyclin B)
and a low molecular weight subunit (p13-Sucl) (Brizuela,
L. <u>et al.</u>, <u>EMBO J.</u> <u>6</u>:3507-3514 (1987); Dunphy, W. <u>et al.</u>,
<u>Cell</u> <u>54</u>:423-431 (1988); Gautier, J. <u>et al.</u>, <u>Cell</u>
<u>54</u>:433-439 (1988); Arion, D. <u>et al.</u>, <u>Cell</u> <u>55</u>:371-378
(1988); Draetta, G. <u>et al.</u>, <u>Cell</u> <u>56</u>:829-838 (1989);
Booher, R. <u>et al.</u>, <u>Cell</u> <u>58</u>:485-497 (1989); Labbe, J-C. <u>et
al.</u>, <u>EMBO J.</u> <u>8</u>:3053-3058 (1989); Meijer, L. <u>et al.</u>, <u>EMBO
J.</u> <u>8</u>:2275-2282 (1989); Gautier, J. <u>et al.</u>, <u>Cell</u> <u>60</u>:487-494
(1990); Gautier, J. and J. Maller, <u>EMBO J.</u> <u>10</u>:177-182
(1991)). cdc2 and related kinases also associate with
other cyclins (Giordana, A. <u>et al.</u>, <u>Cell</u> <u>58</u>:981-990
(1989); Draetta, G. <u>et al.</u>, <u>Cell</u> <u>56</u>:829-838 (1989);
Richardson, H.E. <u>et al.</u>, <u>Cell</u> <u>59</u>:1127-1133 (1989)), and
comprise a family of related enzymes that act at various
stages of the division cycle (Paris, J. <u>et al.</u>, <u>Proc.
Natl. Acad. Sci. USA</u> <u>88</u>:1039-1043 (1990); Elledge, S.J.
and M.R. Spottswood, <u>EMBO J.</u> <u>10</u>:2653-2659 (1991); Tsai,
L-H. <u>et al.</u>, <u>Nature</u> <u>353</u>:174-177 (1991)).

The cdc2/cyclin B enzyme is subject to multiple
levels of control. Among these, the regulation of the
catalytic subunit by tyrosine phosphorylation is the best
understood. In a variety of eukaryotic cell types, cdc2
is one of the most heavily tyrosine phosphorylated

proteins (Draetta, G. et al., Nature 336:738-744 (1988);
Dunphy, W.G. and J.W. Newport, Cell 58:181-431 (1989);
Morla, A.O. et al., Cell 58:193-203 (1989)).
Phosphorylation of the tyrosine 15 and also threonine 14

5   residues of cdc2 is regulated, in part, by the
accumulation of cyclin above a threshold level at which
association with cdc2 occurs (Solomon, M.J. et al., Cell
63:1013-1024 (1990)). Tyrosine phosphorylation inhibits
the cdc2/cyclin B enzyme, and tyrosine dephosphorylation,

10   which occurs at the onset of mitosis, directly activates
the pre-MPF complex (Gautier J. et al., Nature 339:626-629
(1989); Labbe, J.C. et al., EMBO J. 8:3053-3058 (1989);
Morla, A.O. et al., Cell 58:193-203 (1989); Dunphy, W.G.
and J.W. Newport, Cell 58:181-431 (1989); Morla, A.O. et

15   al., Cell 58:193-203 (1989); Gould, K. and P. Nurse,
Nature 342:39-45 (1989); Jessus, C. et al., FEBS LETTERS
266:4-8 (1990)).

       Given the role of cdc2 dephosphorylation in acti-
vation of MPF, there is much interest in the regulation of

20   the cdc2 phosphatase. Genetic studies in fission yeast
have established that the cdc25 gene function is essential
for the initiation of mitosis (Nurse, P. et al., Mol. Gen.
Genet. 146:167-178 (1976). The cdc25 gene product serves
as a rate-determining activator of the cdc2 protein kinase

25   (Russell, P. and P. Nurse, Cell 45:145-153 (1986);
Ducommun, B. et al., Biochem. Biophys. Res. Common.
167:301-309 (1990); Moreno, S. et al., Nature 344:549-552
(1990)). Moreover, the mutant cdc2-F15, whose product
cannot be phosphorylated on tyrosine, bypasses the

30   requirement for cdc25 protein function (Gould, K. and P.
Nurse, Nature 342:39-45 (1989)). Additional work has
suggested that cdc25 is the cdc2 phosphatase. (Kumagai,
A. and W.G. Dunphy, Cell 64:903-914 (1991); Strausfeld, U.
et al., Nature 351:242-245 (1991)) and that cdc25 is the

35   cdc2 phosphatase which dephosphorylates tyrosine and

possibly threonine residues on p34^{cdc2} and regulates MPF
activation. (Dunphy, W.G. and A. Kumagai, <u>Cell 67</u>:189-196
(1991); Gautier, J. <u>et al.</u>, <u>Cell 67</u>:197-211 (1991)).

5 The universal intracellular factor MPF triggers the
G2/M transition of the cell cycle in all organisms. In
late G2, it is present as an inactive complex of tyrosine-
phosphorylated p34^{cdc2} and unphosphorylated cyclin B^{cdc13}.
In M phase, its activation as an active MPF displaying
histone H1 kinase activity originates from the specific
10 tyrosine dephosphorylation of the p34^{cdc2} subunit by the
tyrosine phosphatase p80^{cdc25}. Little is known about the
signals which control or determine timing of MPF
activation and entry into mitosis or about ways in which
those signals can be blocked or enhanced, resulting in
15 inhibition or facilitation of entry into mitosis.

Because the signals that control dephosphorylation of
cdc2 on tyrosine and threonine play a key role in
controlling timing of MPF activation and entry into
mitosis, there is great interest in the proteins which
20 control cdc2 dephosphorylation. Further knowledge of
these proteins and their regulatory functions would be
useful because it would provide a basis for a better
understanding of cell division and, possibly, an approach
to altering how it occurs.

25 <u>Summary of the Invention</u>

For the first time, a key aspect of control of MPF
activation and, thus, entry into mitosis, has been
demonstrated. That is, B-type cyclins have been shown to
activate cdc25 PTPase and a cdc25 protein has been shown
30 to be able to stimulate directly the kinase activity of
pre-MPF, resulting in activation of the M-phase kinase. As
a result, it is now possible to design approaches to
regulating entry into mitosis and, thus, regulate the cell
cycle.

As described herein, Applicant has isolated two
previously undescribed human cdc25 genes, designated cdc25
A and cdc25 B, and has established that human cdc25 is a
multigene family, consisting of at least three members. As
further described herein, cdc25 A and cdc25 B have been
shown to have an endogenous tyrosine phosphatase activity
that can be specifically activated by B-type cyclin, in
the absence of cdc2. It has also been shown for the first
time that cdc25 phosphatases and B-type cyclins interact
directly and that cyclin B is a multifunctional class of
proteins which serve, in addition to their recognized role
as regulatory subunits for M-phase cdc2, a previously
unknown and surprising role as activators of the cdc25
phosphatase. In addition, Applicant has shown that, in
Xenopus, cdc25 levels do not change, either during meiotic
maturation or early embryonic division cycles; that cdc25
physically associates with a cdc2/cyclin B complex in a
cell cycle dependent manner; that the maximal association
between cdc25 and the cdc2/cyclin B complex occurs just
before or at the time of maximal kinase activity (of
cdc2); and that the cdc2 associated with cdc25 is tyrosine
dephosphorylated and active as a kinase. In addition, as
a result of the work described herein, it is now evident
that in Xenopus, cyclin is the only protein that must be
synthesized during each round of activation and inacti-
vation of MPF. It had previously been proposed that
cyclin must accumulate to a critical threshold before
pre-MPF is activated. However, it is reasonable, based on
the work described herein, to suggest that this threshold
marks the point at which sufficient cyclin B has
accumulated to allow activation of the continuously
present cdc25 phosphatase (which, in turn, stimulates
kinase activity of pre-MPF).

As also described herein, a surprising observation
has been made as a result of comparison of the amino acid

sequences of newly discovered cdc25 A and cdc25 B gene
products with known tyrosine protein phosphatases
(PTPases) and other proteins involved in the cell cycle.
That is, it has been shown that the region of cdc25

5   immediately C-terminal to the putative catalytic domain is
not highly related to that of other known PTPases.
Particularly interesting is the fact that this region
within PTPases includes sequence similarity to cyclins,
particularly B-type cyclins, and that cdc25 proteins have

10   no equivalent "cyclin region". The newly found cyclin
region is almost immediately adjacent to the domain
implicated in the catalytic function of the PTPases and
cdc25 protein. As a result of these findings, parti-
cularly the observation that cdc25 protein lacks a motif,

15   shared by cyclin and other PTPases, that may be an
activating domain, it is reasonable to suggest that in the
case of cdc25, the activating domain is provided "in
trans" by intermolecular interaction with cyclin.

As a result of the work described herein, new

20   approaches to regulating the cell cycle in eukaryotic
cells and, particularly, to regulating the activity of
tyrosine specific phosphatases which play a key role in
the cell cycle, are available. Applicant's invention
relates to methods of regulating the cell cycle and,

25   specifically, to regulating activation of cdc2-kinase,
through alteration of the activity and/or levels of
tyrosine phosphatases, particularly cdc25 phosphatase, and
B-type cyclin, or through alteration of the interaction of
components of MPF, particularly the association of cdc25

30   with cyclin, cdc2 or the cdc2/cyclin B complex. The
present invention also relates to agents or compositions
useful in the method of regulating (inhibiting or
enhancing) the cell cycle. Such agents or compositions
are, for example, inhibitors (such as low molecular weight

35   peptides or compounds, either organic or inorganic) of the

catalytic activity of tyrosine specific PTPases
(particularly cdc25), blocking agents which interfere with
the interaction or binding of the tyrosine specific PTPase
with cyclin or the cyclin/cdc2 complex, or agents which
5   interfere directly with the catalytic activity of the
PTPases.

Applicant's invention also relates to cdc25 A, cdc25
B and additional members of the cdc25 multigene family and
to methods and reagents (e.g., nucleic acid probes,
10 antibodies) useful for identifying other members of the
cdc25 family, particularly those of mammalian (e.g.,
human) origin.

Applicant's invention also includes a method of
identifying compounds or molecules which alter (enhance or
15 inhibit) stimulation of kinase activity of pre-MPF and,
thus, alter (enhance or inhibit) activation of MPF and
entry into mitosis. The present method thus makes it
possible to identify agents which can be administered to
regulate the cell cycle; such agents are also the subject
20 of this invention.

The present method makes use of a cell cycle-specific
target and, thus, provides a highly specific mechanism-
based screen for agents (compounds or molecules) which
alter mitosis, particularly antimitotic agents. In the
25 subject method, an agent is assessed for it's effect on the
essential cell cycle-regulating component, cdc25 (e.g.,
cdc25A, cdc25B, cdc25C).

In particular, the agent to be assessed for its
ability to inhibit cdc25 tyrosine phosphatase activity is
30 combined with cdc25 and a substrate of cdc25 tyrosine
phosphatase activity. The resulting combination is
maintained under conditions appropriate for cdc25 to act
upon the substrate. It is then determined whether cdc25
acted upon the substrate when the compound being assessed
35 was present; the extent to which cdc25 acts upon the

substrate in the presence of the compound is compared with the extent to which cdc25 acts on the substrate in the absence of the compound (in comparison with a control). If cdc25 activity is less in the presence of the compound,

5   the compound is an inhibitor of cdc25.

More particularly, a potential antimitotic agent (i.e., an agent to be assessed for an antimitotic effect) is combined with cdc25, which is either cdc25 protein or a fusion protein (e.g., recombinant p80^{cdc25} present in a two-

10 component fusion protein in which cdc25 is joined with a second component, such as glutathione-S-transferase). Subsequently, the effect of the potential antimitotic agent on the phosphatase activity of cdc25 is determined. p80^{cdc25} protein has been shown, as described herein, to

15 have p-nitrophenylphosphate phosphatase activity. Thus, the inhibitory effect of the agent being tested on cdc25 can be assessed using p-nitrophenylphosphate or inactive cyclin/cdc2 as substrate. Results obtained (e.g., the extent of inhibition of cdc25 phosphatase activity) are

20 particularly valuable, since they demonstrate the effect of the agent tested on a target which is particularly well suited for detecting antimitotic agents because of its direct role in controlling entry of cells into M phase.

Brief Description of the Figures

Figures 1A-F are the nucleotide sequence of cdc25 A and the nucleotide sequence of cdc25 B.   Panel A , sequence of cdc25 A cDNA (SEQ ID NO. 1).   Panel B, sequence of cdc25 B (SEQ ID NO. 3). Below the nucleotide sequence is the translation in standard single letter amino acid code.

30 In each sequence, the presumed initiating methionine is underlined. An in-frame stop codon upstream of the initiating AUG codon in the cdc25 A sequence is in bold and in each sequence, the terminating codon is marked by an asterisk.

Figure 2 shows the homology of cdc25 proteins. The amino acid sequences of cdc25 A and cdc25 B were aligned with human cdc25 C (formerly CDC25Hs), string (Stg) and S. pombe cdc25 (25Sp) using the FASTA program. Identical
5 amino acids are boxed. In cases of only two alternative amino acids at a particular site a box is also used. Dashes within the sequences indicate individual amino acid gaps created by the computer to generate optimal alignment.

Figures 3A-B provide proof that human cdc25 A is essential for mitosis. Figure 3A is a graphic representation of the mitotic index of a population of the HeLa cells microinjected at time zero with the affinity-purified anti-cdc25A antibodies. Control cells
15 were microinjected with the IgG fraction of the preimmune serum. Figure 3B is a graphic representation of the estimation of cell numbers in islands of HeLa cells injected at time zero with control or experimental anticdc25A affinity purified antibodies.

Figures 4A-C show activation of cdc25A phosphatase by mitotic cyclins. Human GST-cdc25 A fusion protein was used to assay release of 32p: substrates were tyrosine phosphorylated, reduced carboxamidomethylated, maleylated lyzosyme (RCML) (A); cdc2-derived peptide (B); or PNPP
25 (C). A410 indicates adsorbance at 410 nm.

Figure 5 is a graphic representation of dose-dependent activation of the cdc25 A by cyclin B1. Bars indicate the standard error in three experiments.

Figure 6 shows inhibition of cdc25 phosphatase
30 activity by p13 (Suc1). In the left panel, cdc25 A (10 pmoles) and right panel, cdc25 B (10 pmoles) was used. Bars indicate the standard error in three independent experiments.

Figures 7A-B show the alignment of the cdc25 proteins,
35 PTPases and cyclins and a model of a proposed relationship

between PTPases and the M-phase kinase and cdc25
phosphatase. Panel A depicts the alignment, in which CA
indicates the puative catalytic domain of the cdc25 and
cytoplasmic tyrosine phosphatases, and CR indicates the
5 cyclin related domain, present in tyrosine phosphatases
but absent in cdc25 proteins. Panel B depicts a schematic
representation of the hypothetical relationship between
PTPases, and the M-phase kinase and cdc25 phosphatase.

Figure 8 is a graphic representation demonstrating
10 that Xenopus cdc25 is required for activation of M-phase
kinase. The ammonium sulfate fraction of the prophase
oocyte extract was incubated in the presence of either
PBS-2%BSA (filled·diamonds) preimmune anti-cdc25 serum
(oper circles; open diamonds), or purified anti-cdc25
15 antibody (filled rectangles; open rectangles). In two
cases (open diamonds; open rectangles), soluble
bacterially expressed yeast cdc25 protein (100 mg/ml) was
added (indicated by arrows).

Figure 9 is a graphic representation evidencing
20 periodic physical association of cdc25 and cdc2/cyclin B.
Filled rectangles indicate histone H1 kinase activity of
p13-Sepharose precipitates; open rectangles indicate
amounts of cdc2 found in anti-cdc25 immunocomplexes by
blotting with anti-cdc2 antibody.

25 Figure 10 is a schematic representation of the
control by p80^{cdc25} of activation of inactive pre-MPF (G2)
to active MPF (M phase).

Figure 11 is evidence that the GST-cdc25a fusion
protein dephosphorylates p34^{cdc2} and activates the M phase-
30 specific H1 kinase (MPF).

Figures 12A-B are graphic representation of GST-cdc25-
pNPP phosphatase activity as a function of GST-cdc25A
concentration (Figure 12A) and as a function of duration
of assay (Figure 12B).

Figures 13A-B are graphic representation of GST-cdc25a activity as a function of DTT concentration (Figure 13A) and p-NPP concentration (Figure 13B).

Figure 14 is a graphic representation of the
5 inhibitory effect of sodium orthovanadate on GST-cdc25A tyrosine phosphatase, in which phosphatase activity is expressed as % of activity in the absence of vanadate (mean ±SD).

Detailed Description of the Invention

10 The present invention relates to a method of regulating (inhibiting or enhancing) cell division and to agents or compositions useful for regulating the cell cycle. It further relates to two human genes, referred to as cdc25 A and cdc25 B, encoding tyrosine-specific
15 phosphatases, the encoded tyrosine-specific phosphatases and additional members of the cdc25 multigene family, particularly additional human cdc25 genes, and their encoded products. In addition, the invention relates to a method of identifying agents which alter stimulation of
20 kinase activity and thus alter entry of the cell into mitosis. The present invention also relates to an assay in which cdc25 tyrosine phosphatase, such as cdc25 protein or recombinant human cdc25 tyrosine phosphatase, is used as a cell cycle-specific target to screen for compounds
25 which alter entry into mitosis (passage from late G2 into the M phase). Applicant's invention is based on identification of new cdc25 genes and the discovery that cdc25 proteins interact directly with and are specifically activated by B-type cyclins and activate cdc2 kinase.

30 Applicant has isolated two human cdc25 genes, designated cdc25 A and cdc25 B, and has thus established that human cdc25 is a multigene family of at least three members. The three human cdc25 proteins (cdc25 A, cdc25 B and the previously identified cdc25 protein) have been

shown to have approximately 40% identity in the most
conserved C-terminal region. The cdc25 A and cdc25 B
proteins can be classified as cdc25 proteins by a variety
of independent criteria.

5   As shown herein, the cdc25 A gene product and cdc25 B
gene product have endogenous tyrosine phosphatase activity
in vitro which is stimulated several-fold, in the absence
of cdc2, by cyclin B1 or cyclin B2. As is also shown
herein, stable association occurs between cdc25 A and

10   cyclin B1/cdc2 in human cells, specifically HeLa cells.
These findings indicate that B-type cyclins are
multi-functional proteins which not only are M-phase
regulatory subunits, but also activate the cdc25 tyrosine
phosphatase which, in turn, acts upon cdc2.

15   A region of amino acid similarity between cyclins and
cytoplasmic tyrosine phosphatases has been identified and
shown not to be present in cdc25 phosphatases, suggesting
that the common motif represents an activating domain
which must be provided to cdc25 by cdc25-cyclin B

20   intramolecular interaction. Specifically, visual
comparison of cdc25 A and cdc25 B with known tyrosine
phosphatases (PTPases) and other proteins involved in cell
cycle control resulted in the unexpected observation that
a region of cdc25 immediately C-terminal to the putative

25   cdc25 catalytic domain is not highly related to other
known PTPases and that this newly found motif within the
PTPases includes sequence similarity to cyclins,
particularly of the B-type. Alignment of amino acid
sequences of the cdc25 homologs and a diverse group of

30   protein tyrosine phosphatases (PTPs) demonstrated that a
C-terminal fragment of approximately 200 amino acid
residues is a conserved protein motif which resembles the
proposed catalytic center of viral and mammalian PTPases
(see Example 1 and Figure 2).

Applicant has shown that the two new human cdc25 genes encode proteins functionally related to that encoded by the fission yeast cdc25 (Example 2). One of the human cdc25 genes (cdc25 A) has been shown to act in mitosis in

5 human cells (Example 3), which arrest in a "rounded up" mitotic state after microinjection of anti-cdc25 A antibodies. Thus, Applicant has shown for the first time that the PTPase is necessary for cell division, Applicant has also shown that cell division is inhibited by

10 anti-cdc25 A antibodies, which are, thus, a cytotoxic agent.

Surprisingly, it has also been shown that the endogenous phosphatase activity of cdc25 A and cdc25 B proteins purified from E. coli is directly activated by

15 stoichiometric addition of B-type cyclin, in the absence of cdc2 (Examples 4 and 5), thus showing that B-type cyclins have a multifunctional role in this stage of cell division. This clearly demonstrates specificity between cyclins in their role as activators of cdc25. Until this

20 finding, it has proved difficult to demonstrate differences in substrate specificity among members of the cdc2/cyclin family, although a variety of lines of evidence have suggested that cyclins of different classes have specific roles at particular stages of cell division.

25 The cdc25 A protein has been shown to be present in a complex with both cyclin B1 and cdc2 (Example 5).

Applicant has also determined that Xenopus oocytes contain a relative of cdc25, designated p72, which can directly stimulate the M-phase kinase in vitro and is

30 essential for activation of the M-phase kinase in cell-free lysates. As described herein, the abundance of p72 does not change in Xenopus embryos during the cell cycle. p72 has been shown to directly associate with cdc2/cyclin B in a cell cycle dependent manner, reaching a

35 peak at M-phase. The M-phase kinase which associates with

p72 has been shown to be tyrosine dephosphorylated and catalytically active. As a result, it is reasonable to conclude that cdc25 triggers cdc2 activation by a mechanism which involves periodic physical association

5 between cdc25 and the cyclin B/cdc2 complex, and that it is the association between cdc2/cyclin B and cdc25 which is required. It is also reasonable to conclude that mitotic control can be effected by mechanisms other than transcriptional regulation of the cdc25 gene.

10   As a result of Applicant's findings concerning the role of cdc25 in cell division, an assay is now available in which cdc25 is used as a cell-cycle specific target to screen for compounds which alter a cell's entry into the mitosis phase of cell growth. Results of the assay (i.e.,

15 the ability of the compound being tested to inhibit cdc25) are determined by known techniques, such as colormetrically, by immunoassay techniques or by detecting enzymatic activity (e.g., histone kinase activity).

The following describes Applicant's isolation and

20 characterization of two new human cdc25 genes; demonstration of the multifunctional role of B-type cyclin in mitosis; the unexpected observation of a common amino acid sequence or motif present in PTPases and cyclins but absent in cdc25, and the determination that the motif

25 resembles the proposed catalytic center of viral and mammalian PTPs; demonstration of a specific interaction between cdc25 phosphatases and B-type cyclins; and demonstration that the level of cdc25 in Xenopus oocytes does not change during the cell cycle. As a result of the

30 work described, novel methods and compositions for cell cycle regulation are available, as well as an assay for compounds which alter cell cycle regulation. These methods, compositions, and assay are also described below.

Isolation and Characterization of Two New Human cdc25 Genes Which Are Members of a Multigene Family

Two new human cdc25 genes have been isolated, establishing the fact that in humans, cdc25 is a multigene
5 family that consists of at least three members. The three human cdc25 proteins share approximately 40% identity in the most conserved C-terminal region. The two newly discovered cdc25 genes, cdc25 A and cdc25 B, can be classified as cdc25 proteins by a variety of quite
10 independent criteria. First, they share sequence similarity with other members of the family. Second, cdc25 A and cdc25 B can each rescue a mutant cdc25-22 strain of fission yeast. Third, injection of antibodies prepared against a peptide comprising part of the cdc25 A
15 protein into proliferating HeLa cells causes their arrest in mitosis. Fourth, cdc25 A protein eluted from immunocomplexes can activate the latent histone kinase activity of cdc2. Fifth, both cdc25 A and cdc25 B purified from E. coli display an endogenous tyrosine
20 phosphatase activity.

The cdc25 Multigene Family

As described, it has now been shown that in humans, there are at least three cdc25 genes and possibly more. In fission yeast, only one essential cdc25 gene has been
25 identified to date (Russell, P. and P. Nurse, Cell 45:145-153 (1986)). Likewise, a single essential mitotic B-type cyclin has been described in this yeast (Booher, R. and D. Beach, EMBO J. 7:2321-2327 (1988)). Two mitotic B-type cyclins have been found both in frog and humans
30 (Minshull, J. et al., Cell 56:947-956 (1989)). Presumably, there is some differentiation of function between different members of the cdc25 and B-type cyclin families in vivo. Genetic studies in budding yeast, in which multiple B-type cyclins have been found, give some general

hint that this is the case (Surana, U. et al., Cell
65:145-161 (1991); Ghiara, J.B. et al., Cell 65:163-174
(1991)). However, both cyclin B1 and B2 could activate
cdc25 A in vitro. One might postulate that different
human cdc25 genes activate different cyclin B/cdc2
complexes in vivo and this may explain why injection of
anti-cdc25 A serum into HeLa cells causes arrest in
mid-mitosis, rather than in interphase.

It should be noted that regulation of cdc2 by
tyrosine phosphorylation has currently only been described
with respect to the cdc2/cyclin B enzyme. However, in
certain contexts, it has been possible to substitute
cyclin B with cyclin A (Swenson, K.I., et al., Cell
47:861-870 (1986)); Pines, J. and T. Hunt, EMBO J.
6:2987-2995 (1987)), and indeed human cyclin B2 was
isolated by virtue of its ability to rescue a cn-deficient
strain of budding yeast (Xiong, Y. et al., Cell 65:691-699
(1991)). In the work described herein, cyclin A could not
activate cdc25 A or cdc25 B (not shown). This does not
preclude, however, the existence of undiscovered
cdc25-related phosphatases, that might be specifically
activated by cyclin A. It is also presently unknown
whether relatives of cdc2, such as cdk2 (formerly egl,
Paris, J. et al., Proc. Natl. Acad. Sci. USA 88:1039-1043
(1991); Elledge, S.J. and M.R. Scottswood, EMBO J.
10:2653-2659 (1991)), that can bind cyclin A (Tsai, L-H.
et al., Nature 353:174-177 (1991)), are subject to
regulation by tyrosine phosphorylation and, hence, might
require a cdc25 relative for activation.

Multifuncitonal Role Of B-type Cyclin In Mitosis

A particularly striking observation described herein
is the demonstration that the endogenous phosphatase
activity of cdc25 A and cdc25 B proteins purified from E.
coli can be directly activated by stoichiometric addition

of B type cyclins. Specificity of this effect is shown by
the inability of either cyclin A or cyclin D1 to display
any such stimulation. A variety of lines of evidence
suggest that cyclins of different classes have specific

5 roles at particular stages of the division cycle (Booher,
R. and D. Beach, EMBO J. 6:3441-3447 (1987); Booher, R.
and D. Beach, EMBO J. 7:2321-2327 (1988); Nash, R. et
al., EMBO J. 7:4335-4346 (1988); Hadwiger, J.A. et al.,
Proc. Natl. Acad. Sci. USA 86:6255-6259 (1989);

10 Richardson, H.E. et al., Cell 59:1127-1133 (1989); Cross,
F., Mol. Cell. Biol. 8:4675-4684 (1980); Wittenberg, C. et
al., Cell 61:225-237 (1990); Draetta, G. et al., Cell
56:829-838 (1989); Giordano, A. et al., Cell 58:981-990
(1989); Pines, J. and T. Hunter, Nature 346:760-763

15 (1990); Xiong, Y. et al., Cell 65:691-699 (1991); Lew,
D.J. et al., Cell 66:1-10 (1991); Koff, A. et al., Cell
88:1-20 (1991)). However, it has proved difficult to
demonstrate differences in substrate specificity between
members of the cdc2/cyclin family in vitro, and all known

20 cyclins can rescue a CLN-deficient strain of budding
yeast. The present experiments vividly demonstrate
specificity between different cyclins in their role as
activators of cdc25.

Certain evidence, both genetic and biochemical,

25 suggests that cdc2 is a physiological substrate of cdc25
phosphatases (Gould, K. and P. Nurse, Nature 342:39-45
(1989); Kumagai, A. and W.G. Dunphy, Cell 64:903-914
(1991); Strausfeld, U. et al., Nature 351:242-245 (1991);
Gautier, J. et al., Cell 67:197-211 (1991)). cdc2 was not

30 used as a substrate in the present study because it binds
to cyclins and, thus, potentially becomes altered as a
phosphatase substrate; therefore, the issue of cdc25
substrate specificity has not been addressed directly.
However, the finding of activation of cdc25, specifically

35 by B-type cyclins, strengthens the conclusion that

cdc2/cyclin B is the relevant substrate _in vivo_.
Demonstration of activation of cdc25 when artificial
PTPase substrates were used leads to the conclusion that
cyclins are able to interact with cdc25 in the total

5   absence of cdc2 protein. _In vivo_, it is expected that
this interaction occurs in the context of the cdc2/cyclin
B pre-MPF complex. The above-described work demonstrates
that B-type cyclins have at least two roles. First, they
bind stoichiometrically with cdc2 to regulate the

10 substrate specificity (Draetta, G. _et al._, _Nature_
_336_:738-744 (1989); Brizuela, L. _et al._, _Proc. Natl. Acad._
Sci. USA 86:4362-4366 (1989)) and the intracellular
localization of the catalytic subunit (Booher, R.N. _et_
_al._, _Cell 58_:485-497 (1989)). Second, they appear to have

15 an independent function: the activation of cdc25 PTPase.
    Genetic studies in fission yeast and Drosophila
indicate that cdc25 is a dose-dependent activator of
mitosis (Russell, P. and P. Nurse, _Cell 45_:145-153 (1986);
Edgar, B.A. and P.H. O'Farrell, _Cell 57_:177-187 (1989)),

20 whereas the cdc13 encoded B-type cyclin is essential for
M-phase, but does not serve as a dose-dependent activator.
Indeed, in many cell types, including the fission yeast,
B-type cyclins accumulate and associate with cdc2 long
before the tyrosine dephosphorylation event at the onset

25 of M-phase (Booher, R.N. _et al._, _Cell 58_:485-497 (1989)).
In some somatic cell types, the cdc25 gene is under
transcriptional control, and very probably the cdc25
protein itself is regulated in a variety of ways that are
not presently understood. In the early embryos of Xenopus,

30 a somewhat different situation holds. As shown herein,
the abundance of cdc25 is invariant during the cell cycle.
Cyclin is the only protein that has to be synthesized
during each round of activation and inactivation of MPF
(Murray, W.W. _et al._, _Nature 339_:280-286 (1989)). It has

35 been proposed that, in this context, cyclin must

accumulate to a critical threshold before pre-MPF is
activated (Evans, T. et al., Cell 33:389-396 (1983);
Pines, J. and T. Hunt, EMBO J. 6:2987-2995 (1987);
Minshull, J. et al., Cell 56:947-956 (1989); Murray, A.W.
5   and M.W. Kirshner, Nature 339:280-286 (1989)). Based on
work described herein, it appears that this threshold
marks the point at which sufficient cyclin has accumulated
to allow activation of the continuously present cdc25
phosphatase.

10   The present findings may throw light on the long
obscure phenomenon of MPF autoactivation. If a small
amount of MPF is injected into a frog oocyte, a much
larger amount can subsequently be retrieved (Masui, Y. and
C.L. Markert, J. Exp. Zool. 171:129-146 (1971); Smith,
15   L.D. and R.E. Ecker, Dev. Biol. 25:232-247 (1971)). The
present work shows that in this situation, the abundance
of cdc2, cyclin B and cdc25 do not change (Gautier, J. and
J. Mailer, EMBO J. 10:177-182 (1991); see also Example
11). It has been implicitly assumed that active
20   cdc2/cyclin B phosphorylates some protein (possibly cdc25
itself), causing the activation of cdc25 and, thus leading
to further activation of pre-MPF. This may be correct,
but if cyclin B directly activates cdc25 in the absence of
cdc2, as shown herein, all of the elements needed for an
25   autoactivation loop exist among the cdc2, cyclin B and
cdc25 proteins themselves.

A Common Motif in PTPases and Cyclins

Alignment of the cdc25 proteins, PTPases and cyclins
was performed, as shown in Figure 7A. Tyrosine phos-
30   phatases were aligned with each other as described in
Guan, K. et al., (Nature 350:359-362 (1991)) and cdc25
proteins as described in Gautier, J. et al., (Cell
67:197-211 (1991)). The cyclin alignment was done by
visual inspection. Only identity or similarity (V or I)

within at least three members of one gene family and a
minimal of two members of other family is boxed. Visual
comparison of cdc25 A and B with known tyrosine PTPases,
and also other proteins involved in cell cycle control,
5   resulted in the following unexpected observations. First,
the region of cdc25 that is immediately C-terminal to the
putative catalytic domain (CA) is not highly related to
other known PTPases, such as cytoplasmic PTPases from
higher eukaryotes and the vaccinia virus serine-tyrosine
10 phosphatase (VH-I, Guan, et al., Nature 350:359-362
(1991); Figure 7A). Second and more interestingly, this
region within the PTPases was found to contain sequence
similarity to cyclins, particularly of the B-type (Figure
7A). The similarity was detected immediately at the
15 junction of the so-called cyclin-box and included some
nearly invariable residues among cyclins. The alignment
in Figure 7A optimizes the similarities between cdc25
proteins and PTPases, and also between PTPases and
cyclins, but ignores the much greater homology within each
20 of the three groups of proteins. In the region of
similarity between PTPases and cyclins, referred to as the
cyclin region (CR), there is no equivalent in the cdc25
proteins.

The newly found motif lies almost immediately
25 adjacent to the domain (V/IXHCXXXXR), that has been
directly implicated in the catalytic function of the
PTPases and cdc25 protein (Krueger, N.S. et al., EMBO J.
9:3241-3252 (1990); Guan, K. and J.E. Dixon, Science
249:553-556 (1990); Guan, K. et al., Anal.Biochemistry
30 192:262-267 (1991); Gautier, J. et al., Cell 67:197-211
(1991)). This finding allows the following speculation.
The catalytic activity of the other PTPases is consider-
ably greater than that of cdc25, at least as determined in
this study. cdc25 lacks the motif that is shared by
35 cyclins and other PTPases. This motif may be an acti-

vating domain which, in the case of cdc25, is provided in "trans" by intermolecular interaction with cyclin (Figure 7B), although in most PTPases it functions in "cis".

There is some similarity between PTPases and all of the classes of cyclin, whereas only B-type cyclins can activate cdc25. It is apparent, however, that the similarity is greatest between PTPases and cyclins of the B class. The differences between the various classes of cyclins within this region might be related to the specific ability of B but not A or D-type cyclins to activate cdc25 A.

Specific Interaction of cdc25 with Cyclin B

As shown in Example 13, cdc25 stably associates with a cdc2 complex and this interaction is periodic during the division cycle of Xenopus embryos. Human cyclin B1 is found in the complex with cdc25 A, as described in Example 5. It seems likely that the periodicity of the interaction between cdc25 and cdc2 is mediated at least in part by periodic accumulation and degradation of cyclin during the cell cycle.

As described herein, it has been established that cdc25 can function as an enzyme with respect to RCML, PNPP and cdc2 derived peptide substrates. A low observed catalytic rate was evident and may reflect the use of RCML or peptide as an artificial substrate. However, it is not clear what catalytic rate is required in vivo. If cdc25 does indeed associate with cdc2/cyclin B as suggested herein (Example 9 and Figure 7), the PTPase may not function in a conventional catalytic reaction, but rather only after formation of a cdc25/cyclin B/cdc2 complex. Under such conditions, the catalytic reaction is essentially intramolecular and Michaelis/Menten kinetics do not pertain.

Inhibition by p13 of Human cdc25 Phosphatase Activity

The p13 protein encoded by the suc1 gene is an essential subunit of the cdc2 protein kinase. The gene was isolated by virtue of its ability to rescue a fission

5 yeast cdc2-33 allele on a multicopy plasmid (Hayles, J. et al., EMBO J. 5:3373-3379 (1986)). However, overexpression of the gene is inhibitory for mitosis (Hindley, J. et al., Mol. Cell. Biol. 7:504-511 (1987); Hayles, J. et al., Mol Gen. Genet. 202:291-293 (1986)). In vitro, p13 can

10 inhibit activation of pre-MPF (Dunphy, W. et al., Cell 54:423-431 (1988); Dunphy, W. and J.W. Newport, Cell 58:181-431 (1989)).

The present work may clarify two previously confusing issues related to these observations. First, p13 can bind

15 to cdc2 in the absence of cyclins (Brizuela, L. et al., EMBO J. 6:3507-3514 (1987); see also Example 6), but activation of cdc2/cyclin B that is pre-bound to p13-sepharose can be inhibited by excess exogenous p13 (Jessus, C. et al., FEBS LETTERS 266:4-8 (1990)). By

20 contrast, fully activated cyclin B/cdc2 is not inhibited by excess p13 (Dunphy, W. et al., Cell 54:423-431 (1988); Arion, D. et al., Cell 55:371-378 (1988); Maijer, L. et al., EMBO J. 8:2275-2282 (1989)). This suggests, as previously proposed (Jessus, C. et al., FEBS LETTERS

25 266:4-8 (1990)), that there are at least two binding sites for p13. One is presumably a high affinity binding site on cdc2 itself, that accounts for the extraordinary efficiency of p13-sepharose chromatography. The other site, of lower affinity requiring p13 in the 20 micromolar

30 range, does not affect fully activated cdc2/cyclin B, but can inhibit activation of pre-MPF. Because direct inhibition of cdc25 A endogenous phosphatase activity by p13, in the total absence of cdc2, has been observed (Example 6), it is reasonable to attribute the second

35 binding site not to cdc2, but to cdc25. This is probably

an unstable interaction, quite unlike that between p13 and cdc2. A schematic representation of the hypothetical relationship between PTPases, the M-phase kinase and cdc25 phosphatase, is shown in Figure 7B. The association

5   between cdc2 and p13, and between cyclin and cdc2, is well documented. The interaction of cdc25 and cyclin is also proposed here, p13 is proposed to have a low affinity interaction with cdc25. CA is the catalytic domain of PTPases and CR is a region of similarity between PTPases

10 and cyclins.

Second, there has been some dispute concerning the inhibition of cdc25 by p13 in different experimental contexts. In some cases, p13 has been inhibitory (Gautier, J. et al., Cell 67:197-211 (1991)) and in other

15 cases, it has not (Kumagai, A. and W.G. Dunphy, Cell 64:903-914 (1991)). As described herein under the conditions used, cdc25 A is inhibited by p13, and cdc25 B is not. The two proteins have many regions of structural dissimilarity that could readily account for this effect.

20 cdc25 Does Not Change in Abundance During the Cell Cycle

Surprisingly, the Xenopus cdc25 does not oscillate in abundance, either during meiotic maturation, or during the early embryonic division cycles. The protein does, however, physically associate with the cdc2/cyclin B

25 complex in a cell cycle dependent manner (see Examples 5 and 10). Maximal association is found just before or at the time of maximal kinase activity (see Examples 11 and 13, and Figure 9). The cdc2 that is associated with cdc25 is tyrosine dephosphorylated and active as a histone H2

30 kinase. The association between cdc25 and the cdc2/cyclin B complex could be mediated either by cdc2 or by cyclin B. As described herein, B-type cyclins were shown to be able to directly activate the intrinsic PTPase activity of cdc25 proteins in the absence of cdc2. This suggests that

the interaction between cdc25 and the cdc2/cyclin B complex is probably mediated by cyclin.

These results bear upon the mechanism by which cdc2 becomes activated at M-phase. cdc25 acts in mitosis to cause the tyrosine dephosphorylation of cdc2, as described herein. The demonstration of direct physical association between cdc25 and the cdc2/cyclin B complex is entirely consistent with this hypothesis. The finding that approximately 5% of cdc2 associates with cdc25 at M-phase raises certain questions. It is possible that one molecule of cdc25 binds to cdc2/cyclin B, activates the kinase and then dissociates to repeat the process in a conventional catalytic mechanism. Alternatively, a single molecule of cdc25 might activate only a single molecule of pre-MPF in a stoichiometric mechanism. Only a fraction of the total amount of cdc2 (10% of the cellular cdc2 content, as described in Kobayashi A.H. et al., J. Cell Biol. 114:755-765 (1991)) is bound to cyclin B and activated at M-phase in Xenopus eggs. The finding that only 5% of total cdc2 is associated with cdc25 at mitosis might reflect the relatively low abundance of cyclin B compared.to cdc2, if the interaction is mediated by cyclin B. This is confirmed by the fact that, in comparison to the 5% cdc25-associated cdc2, a larger amount of cyclin B2 is found in association with cdc25 (17% of the full cellular amount of cyclin B2). Moreover, a considerable fraction of cdc25 is involved in this association (20% of the cellular content).

Identification of Additional cdc25 Genes and Cell Cycle
Regulation by the Present Invention

Using methods described herein, such as in Examples 1 and 7, additional members of the human cdc25 gene family and cdc25 genes in other organisms can be identified and isolated; the encoded products can be identified as well.

For example, all or a portion of the nucleotide sequence
of the cdc25 A gene or the cdc25 B gene (see Figure 1) can
be used in hybridization methods or amplification methods
known to those of skill in the art (Sambrook, et al.,

5   Molecular Cloning: A Laboratory Manual, Cold Spring Harbor
Laboratory, NY (1989)). For example, a nucleotide
sequence which is all or a portion of the cdc25 A gene or
the cdc25 B gene can be used to screen a DNA library of
human or nonhuman origin for additional cdc25 genes. DNA

10 sequences identified in this manner can be expressed and
their products analyzed for tyrosine specific phosphatase
activity, such as by the methods described herein (see
Experimental Procedures and Example 2). Hybridization
conditions can be varied as desired. If a nucleotide

15 sequence which is exactly complementary to the probe used
is to be isolated, conditions of either high or low
stringency can be used; if a nucleic acid sequence less
related to those of the probe is to be identified,
conditions of lower stringency are used. The present

20 invention includes the cdc25 A and cdc25 B genes and
equivalent cdc genes; equivalent genes, as used herein,
are nucleic acid sequences which hybridize to all or a
portion of the cdc25 A or cdc25 B gene or a complement of
either gene, and encode a tyrosine PTPase which has

25 substantially the same catalytic function as the cdc25 A
or cdc25 B gene product. The polymerase chain reaction
and appropriately designed primers can also be used to
identify other cdc25 genes. Alternatively, an anti-cdc25
A or anti-cdc25 B antibody can be used to detect other

30 (recombinant) cdc25 gene products expressed in appropriate
host cells transformed with a vector or DNA construct
thought to encode a cdc25 product. The cdc25 A gene,
cdc25 B gene and equivalent cdc genes which are the
subject of the present invention include those obtained

35 from naturally occurring sources and those produced by

genetic engineering (cloning) methods or by synthetic methods. These genes can be used to produce the encoded cdc25 A, cdc25 B or other cdc25 gene product, which can, in turn, be used to produce antibodies specific for the
5   product or to regulate cell cycle activation (cdc2 kinase activation), as described below.

The present invention also includes PTPase genes which encode PTPases which are related to cdc25 PTPases but are specifically activated by a non-B type cyclin
10   (e.g., by cyclin A, cyclin D). These PTPases are referred to herein as cdc25-related PTPases and their activation by a cyclin, their ability to activate cdc2 or another molecule and their role in regulation of the cell cycle can be assessed using the methods described for
15   determining the role of cdc25.

The present invention also provides a method by which the level of expression or activity of cdc25 PTPases in a cell can be determined and assessed (i.e., to determine if they increased, decreased or remained within normal
20   limits). Because the cdc25 gene is increased (overexpressed) in certain tumor types, the present invention also provides a method of diagnosing or detecting overexpression related to those tumor cell types. In the method, a gene probe to detect and quantify
25   the cdc25 gene in cells, or antibodies specific for the cdc25 PTPase can be used.

Assay for Compounds Which Alter cdc25 Function/Entry into Mitosis

A method of inhibiting activation of cdc25 PTPases,
30   activation of cdc2 kinase(s) and, thus, initiation of mitosis (cell division) is also possible. For example, activation of cdc25 PTPase is inhibited (reduced or prevented) by introducing into cells a drug or other agent which can block, directly or indirectly, complexing of

cdc25 with cyclin B or the cyclin B/cdc2 complex and, thus, directly block activation of the cdc25 and indirectly block activation of the cdc2 kinase. In one embodiment, complex formation is prevented in an indirect

5 manner, such as by preventing transcription and/or translation of the cdc25 DNA and/or RNA. This can be carried out by introducing into cells antisense oligonucleotides which hybridize to the cdc25-encoding nucleic acid sequences, and thus prevent their further

10 processing. It is also possible to inhibit expression of the cdc25 product by interfering with an essential cdc25 transcription factor. Alternatively, complex formation can be prevented by degrading the cdc25 gene product(s), such as by introducing a protease or substance which

15 enhances their breakdown into cells. In either case, the effect is indirect in that a reduced quantity of cdc25 is available than would otherwise be the case. In another embodiment, activation of cdc25 PTPase is inhibited by interfering with the newly identified region of cyclin

20 which has been shown to share sequence similarity with a region present in other PTPases, but not present in cdc25, and which appears to be provided to cdc25 in trans by intermolecular interaction with cyclin.

In another embodiment, activation of cdc25 PTPase is

25 inhibited in a more direct manner by, for example, introducing into cells a drug or other agent which binds the PTPase and prevents complex formation with cyclin (and, thus, prevents PTPase activation). Alternatively, a drug or other agent which interferes in another manner

30 with the physical association between cyclin and the PTPase (e.g., by intercalation), or which disrupts the catalytic activity of the enzyme can be introduced into cells. This can be effected, for example, by use of antibodies which bind the PTPase or the cyclin, or by a

35 peptide or low molecular weight organic or inorganic

compound which, like the endogenous type B cyclin binds
the cdc25 PTPase, but, unlike type B cyclin does not
result in activation of the enzyme or results in its being
disabled or degraded. Peptides and small organic
5   compounds to be used for this purpose can be based on
analysis of the amino acid sequences of B type cyclins or
of the amino acid sequences of the cdc PTPase(s) involved.
They can be designed, for example, to include residues
necessary for binding and to exclude residues whose
10 presence results in activation. This can be done, for
example, by systematically mapping the binding site(s) and
designing molecules which recognize or otherwise associate
with the site(s) necessary for activation, but do not
cause activation. One site of particular interest for
15 this purpose is the region which, as described above, is
missing in cdc25 PTPases and appears to be provided in
trans by intermolecular binding of the cdc25 product and
type B cyclin. At least three possible approaches are
possible in this instance. First, a molecule (e.g., a
20 peptide which mimics the binding site on type B cyclin for
cdc25) can be introduced into cells; the molecule then
binds cdc25 and blocks its interaction with cyclin.
Second, a molecule mimicing the region of cdc25 which
binds the type B cyclin molecule can be introduced into
25 cells; the molecule then binds cyclin and blocks the
cdc25-cyclin complex formation. Third, a molecule which
inhibits or inactivates the putative activating domain on
type B cyclin can be introduced into cells, thus
preventing activation of the cdc PTPase.   .
30   In another embodiment, inhibitors of the catalytic
activity of cdc25 PTPase are introduced into cells. Such
inhibitors are low molecular weight agents, such as
peptides and inorganic or organic compounds.
         The present invention also includes a method of
35 screening compounds or molecules for their ability to

inhibit the function of cdc25 protein or the binding of the cdc25 protein with the cyclin/cdc2 complex. For example, cells as described herein, in which a cdc25 gene is expressed, can be used. A compound or molecule to be
5   assessed for its ability to inhibit cdc25 protein function or binding to the cyclin/cdc2 complex is contacted with the cells, under conditions appropriate for entry of the compound or molecule into the cells. Inhibition of the cdc25 protein or of complex formation will result in
10 arrest of the cells or a reduced rate of cell division. Comparison with cell division of an appropriate control (e.g., the same type of cells without added test drug) will demonstrate the ability or inability of the compound or molecule to inhibit the cyclin. Alternatively, an in
15 vitro assay can be used to test for compounds or molecules able to inhibit cdc25 PTPases or their binding to the cyclin/cdc25 complex. In this in vitro assay, the three components (cdc25 PTPase, cyclin and cdc2 (the latter two either individually or as a cyclin/cdc2 complex such as
20 inactive cyclin/cdc2 complex from interphase cells) are combined with a potential cdc25 inhibitor. The activity of the potential inhibitor is assessed by determining whether cdc25 binds cyclin or cyclin/cdc2 complex or whether cdc2 is activated, as evidenced by histone kinase
25 activity. This method can make use of the teachings of Jessus et al. (FEBS Letters 66:4-8 (1990)) and DuCommun and Beach (Anal. Biochem. 187: 94-97 (1990)), the teachings of which are incorporated herein by reference. For example, in an assay for cdc25 inhibitors, inactive
30 cyclin/cdc2 complex can be placed in the wells, cdc25 and a test compound or molecule added to wells and cdc2 activation assessed. In the presence of a cdc25 inhibitor, cdc2 activation will be prevented or reduced (less than would occur in the absence of the test compound
35 or molecule).

Existing compounds or molecules (e.g., those present in fermentation broth or a chemical "library") or those developed to inhibit the cyclin activation of its protein kinase can be screened for their effectiveness using this method. Drugs which inhibit cdc25 protein catalytic activity, inhibit complex formation or degrade or otherwise inactivate cdc25 are also the subject of this invention.

The present invention also includes an assay in which cdc25 tyrosine phosphatase, such as cdc25 protein or recombinant human cdc25 tyrosine phosphatase, is used to screen for compounds which alter entry into mitosis (passage from late G2 into the M phase of the cell cycle). In one embodiment of the assay, a colorimetric assay can be used to determine the ability of compounds to inhibit the cdc25 tyrosine phosphatase, which is an activator of the protein kinase MPF. As described herein, a glutathione-S-transferase/cdc25A tyrosine phosphatase fusion protein produced in _Escherichia coli_ and purified displays a phosphatase activity towards p-nitro-phenylphosphate. This fusion protein, designated GST-cdc25A, has been used to assess the inhibitory effect of compounds on cdc25 phosphatase activity. In a similar manner, as also described herein, other fusion proteins can be produced and used in the same or a similar assay format. These fusion proteins can differ from GST-cdc25A in either or both of their components. For example, a component other than GST (e.g., maltase binding protein) can be included in the fusion protein with cdc25A. Alternatively, another member of the cdc25 family (e.g., cdc25B, cdc25C) can be the tyrosine phosphatase component. In another embodiment, cdc25 protein is used.

The present method is a simple and rapid screening test which, in one embodiment, uses a fusion protein such as recombinant p80^{cdc25}, assayed through its

p-nitrophenylphosphate phosphatase activity, as a target to test for potential antimitotic compounds. The method has been carried out as a rapid calorimetric microtitration plate assay to test compounds currently

5   used in cancer therapy, and a compound recognized to be a tyrosine phosphatase inhibitor. The therapeutic compounds tested did not display an ability to inhibit cdc25, in the assay as described; the reported tyrosine phosphatase inhibitor (vanadate) was shown, however, to totally

10 inhibit cdc25. Thus, the present method has been shown to be useful in identifying compounds which inhibit an essential cell cycle-regulating component; it provides a highly specific screen for antimitotic drugs.

In one embodiment of the present method, a fusion

15 protein which includes cdc25 is combined, under appropriate conditions, with: 1) an agent to be assessed for its effects on cdc25 and, thus, on passage from late G2 into the M phase; and 2) an appropriate cdc25 substrate, such as p-nitrophenylphosphate or inactive

20 cdc2/cyclin B. The resulting combination is maintained for sufficient time for cdc25 to act upon the cdc25 substrate and the reaction is terminated (e.g., by gross alteration of the pH of the combination). Phosphatase activity of the combination is determined using a known

25 technique, such as by measuring the optical density of the combination and comparing it with a predetermined standard or a control (e.g., a predetermined relationship between optical density and extent of cdc25 inhibition or a combination which includes the same components as the

30 "test" combination except for the agent being assessed).

The fusion protein used in the present method can be produced by known genetic engineering techniques, as described in Example 14. That is, a DNA or RNA construct encoding the fusion protein is introduced into an

35 appropriate host cell, in which the construct is

expressed, thus producing the fusion protein. The fusion protein is separated (and, preferably, purified) from the host cell and used in the assay. Alternatively, the fusion protein can be produced by joining the two

5 separately produced components. As described in Example 15, a fusion protein in which the two components are glutathione-S-transferase and human cdc25A has been produced and used in the subject method.

In a second embodiment, cdc25 protein, such as

10 cdc25A, cdc25B or cdc25C protein, can be used in the subject method. In this embodiment, cyclin/cdc2 can be used as the cdc25 substrate; an agent to be tested is combined with cdc25 protein and cyclin/cdc2 and the tyrosine phosphatase activity of cdc25 is assessed, as

15 described above. Results are compared with a predetermined standard or with a control (see Example 14).

The cdc25 substrate used can be any synthetic or naturally-occurring substance toward which cdc25 demonstrates phosphatase activity. In the embodiment

20 described herein, the cdc25A substrate used is p-nitrophenylphosphate. Other substrates which can be used include peptides that mimic the site of cdc2 phosphorylation or the full inactive cdc2/cyclinB pre-enzyme complex. Others can be identified by using known

25 methods of determining phosphatase activity.

Agents to be tested for their ability to alter cdc25 tyrosine phosphatase activity can be those produced by bacteria, yeast or other organisms, or those produced chemically. The compounds tested herein, as described in

30 Exmaple 18, included 15 drugs currently used in cancer therapy and vanadate, a recognized tyrosine phosphatase inhibitor. The 15 therapeutic agents showed no inhibitory activity. In contrast, vanadate was shown to totally inhibit GST-cdc25A phosphatase. The present method is

35 useful to identify agents potentially effective as

antiproliferative agents and agents which are useful in treating or preventing inflammation or psoriasis, or other diseases relating to cell proliferation.

The present invention will now be illustrated by the
5   following examples, which are not intended to be limiting in any way.

EXPERIMENTAL PROCEDURES

The following experimental procedures were used in carrying out the work described in Examples 1-6.
10   Three highly degenerate primers corresponding to the consensus cdc25 protein sequence were designed taking into account homology between the S. pombe cdc25, Drosophila string and S. cerevisiae mihl gene products. 5'. degenerate primers corresponding to the amino acid sequence IIDCRT/FP
15   (or E) Y E (SIC-1: ATIATIGATTGCCGITA/TCCCITAC/TGA and SIC-2: ATIATIGATTGCCGITA/TCGAITAC/TGA) (SEQ ID NO. 5) and a 3' primer corresponding to the amino acid sequence I/V F H C E F (ST-C: A/TA/GAAC/TTCA/GCAA/GTGA/GAAA/G/TA), (SEQ ID NO. 6) where I corresponds to inosine, were prepared.
20   The 50 ml PCR reaction mixture contained 50 mM KCl; 10 mM TrisHCl(pH 8.3); 1.5 mM $MgCl_2$; 0.01% gelatin; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 0.5 unit of Thermus aquaticus (AmpliTaq DNA polymerase (Perkin-Elmer/Cetus)), 2 mM each of the 5' primers (SIC-I and SIC-2)) 5 mM of the
25   3' primer (ST-C) and 100 mg of human N-Tera cells cDNA library made in ggt10 by Jacek Skowronski (Cold Spring Harbor Laboratory). Four cycles of 94°C for 1 min, 40°C for 3 min and 72°C for 1 min were performed in a DNA thermal cycler (Perkin-Elmer/Cetus). The reaction
30   products were separated on the 2% agarose gel and the expected size (approximately 160 bp) fragments were subcloned into SmaI-digested pBluescript SK(-) vector (Stratagene, La Jolla, CA). Nine clones were sequenced, with the sequence clearly indicating cloning of cdc25

homologues. Two different PCR products were detected:
one of them was almost identical to recently cloned human
cdc25 homologue (CDC25Hs, Sadhu, K. et al., Proc. Natl.
Acad.Sci.USA 87:5139-5143 (1990)), and the other

5   corresponded to a previously uncharacterized cDNA, here
called cdc25 A. The N-Tera cdc25 A PCR-derived clone
(p5w1) was used to screen the human N-Tera cell library at
low stringency. After plaque purification, inserts of
nine positive clones were subcloned into the EcoRI site of

10 the pBluescript SK(-) plasmid. Inserts from two phages
containing the entire open reading frame of the cdc25 A
cDNA were analyzed by restriction mapping (plasmids 4g1.3
and 211.1, containing inserts of 2.4 and 3.9 kb). Plasmid
4g1.3 contained a deletion of 1.4 kb at the 3'

15 untranslated region of the cDNA and was chosen for
complete sequencing. Sequence analysis was performed on
both strands using a chain termination method on an
automated sequencing system (Applied Biosystems 373A).
        Further analysis indicated that one of the original

20 nine phage clones corresponded to a different cdc25
homolog; this is designated cdc25 B. This phage gave rise
to two EcoRI fragments (0.9 and 1.5 kb) but did not
represent a whole open reading frame. In order to obtain
a complete cDNA, the same library was screened with the

25 0.9 kb EcoRI fragment and an insert representing a
complete cDNA (3.0 kb) was subcloned via partial digestion
with EcoRI into the pBluescript SK(-) vector. This was
used for sequencing.

Production of Antipeptide Antiserum to Human cdc25 A and
30 CDC25Hs
        Peptides corresponding to the amino acid sequence
CQGALNLYSQEELF-NH$_2$ (#143)(CDC25Hs or cdc25 C) and
CKGAVNLHMEEEVE-NH$_2$ (#144)(cdc25 A) were synthesized at the
Cold Spring Harbor Laboratory protein core facility,

HPLC-purified and coupled to keyhole limpet hemocyanine
(KLH) and bovine serum albumin essentially as described
(Draetta, G. et al., Nature 336:738-744 (1988)). Rabbits
were injected with 200 mg of KLH-peptide conjugate every
5 three weeks. Positive sera were obtained after three
booster injections. Antibody (K143 and K144) were
affinity purified on the BSA-peptide conjugates coupled to
the CNBr-Sepharose (Pharmacia, Sweden) according to the
manufacturer's instructions. No crossreactivity between
10 peptide #134 and K144 antiserum with the other peptide was
detected.

Rescue of the Fission Yeast cdc25 Temperature Sensitive
Mutant
    A 2.0 kb NcoI-BamHI fragment encoding amino acids
15 1-526 of human cdc25 A from the p4gl.3 plasmid were
subcloned into NcoI-BamHI digested pARTN, resulting in the
pARTN-cdc25 A construct harboring human cdc25 A cDNA in
sense orientation to the constitutive adh promoter. pARTN
is derived from the pART3 (McLeod, et al., 1987) by
20 ligation of an NcoI linker (New England Biolabs) into the
SmaI site. An 2.4 kb SmaI fragment from the p4xl.2
plasmid encoding amino acids 32-566 was subcloned into
SmaI digested pART3 vector (containing LEU2 marker)
resulting in pARTN-cdc25 B cDNA. Both plasmids were
25 transformed into S. pombe h+cdc25-22 leul-32 (SP 532)
strain. Leu+ transformants were obtained at 26°C.

Cell Culture, Immunoprecipitation
    HeLa cells (obtained from the ATCC) were grown at
37°C in Dulbecco modified Eagle's media (DMDM) supple-
30 mented with 10% fetal calf serum. For labelling, cells
were washed with methionine minus media (Gibco) and
supplemented with 1mci/ml ^{35}S-methionine (Translabel, ICN)
for 6-8 hours. Cells were lysed essentially as described

(Draetta, G. et al., Nature 336:738-744 (1988)) or in the
EB buffer (80 mM glycerophosphate, 15 mM MgCl$_2$, 20 mM
EGTA, 1 mM DTT), supplemented with protease inhibitors
(0.5 mM PMSF, 1 mg/ml of aprotinin, pepstatin,

5   chymostatin, leupeptine, 30 mg/ml of TPCK, 15 mg/ml
benzimidine). Lysates were precleared with protein
A-Sepharose beads (Pharmacia) (20 ml of the 1:1 slurry);
anti-human cdc25 A antiserum (K144) were added (1-5 ml);
and after 8-10 hours immune complexes were precipitated

10 with protein A-beads (20 ml of the 1:1 slurry). Beads
were washed four times with the lysis buffer and
resuspended in 20 ml 2x sample buffer (Laemmli, U.K.
Nature 227:680-685 (1970)). Immunoprecipitated proteins
were resolved on the 10% polyacrylamide gels containing

15 SDS, and visualized by the autoradiography of the dried
gel slabs (Anderson, S.J. et al., J. Virol. 51:730-741
(1984)). p13 beads were prepared and used to precipitate
p34^{cdc2} from HeLd as described earlier (Brizuela, L. et al.,
EMBO J. 6:3507-3514 (1987)).

20 Bacterial Expression of the cdc25 A and cdc B Phosphatase
Assay

      A plasmid containing the entire open reading frame of
human cdc25 A was digested with Ncol (at amino acid 1),
blunt ended with T4 DNA polymerase, heat inactivated,

25 extracted with phenolchlorophorm, ethanol precipitated and
digested with EcoRI. The resultant 2.0 kb fragment was
gel-purified and ligated into pGEX-2T Smal/EcoRI digested
vector. Resultant plasmid upon transformation into
bacteria gave rise to a 90 kd IPTG-inducible protein.

30 Expressed fusion protein was recovered as described
(Smith, D.B. and K.S. Johnson, Gene 67:31-40 (1988)) on
glutathione-Sepharose beads (Pharmacia), and eluted with 5
mM freshly prepared glutathione in 50 mM TrisHCl, 50 mM
NaCl, 0.1 mM EDTA, 1 mM DTT, at pH 8.0. For expression of

cdc25 B, plasmid p4xl.2 was cut with XbaI, then with SmaI
(partially) and the 2.4 kb fragment was subcloned into
SmaI/XbaI cut pGEX-KG vector (Guan, K. and J.E. Dixon,
Science 249:553-556 (1991)). Expression of this construct
5 resulted in IPTG-dependent synthesis of the 88 kD
GST-cdc25 B fusion protein. Phosphatase activity of the
purified cdc25 A protein (4.5 mg or 50 pmoles) was assayed
in 0.5 ml 20 mM Tris HCl, pH 8.0, 1 mM EDTA, 0.1%
b-mercaptoethanol, 20 mM p-nitrophenylphosphate (PNPP).
10 Absorbance at 410 nm was determined
using a molar absorptivity of $1.78 \times 10^4 \, M^{-1} \, cm^{-1}$ to calculate
the concentration of the p-nitrophenolate ion generated in
the assay. For cdc25 B the assay was performed in the
same buffer except at pH 8.8.
15   Reduced carboxamidomethylated and maleylated lysozyme
(RCML) was obtained from N. Tonks in a ^{32}p-tyrosine
phosphorylated form. Approximately 50% of the protein was
phosphorylated. ^{32}P-labeled RCML was used in the
phosphatase assay in 50 mM Tris HCl, pH 8.0, 50 mM NaCl,
20 0.1 mm EDTA, 1 mM DTT at a final phosphate concentration
of 10-30 mM. Reactions (30-50 ml) were performed at 30°C
for 10 or 20 min, and after addition of the fatty acid
free bovine serum albumin (BSA, Sigma) to 2 mg/ml,
proteins were precipitated with 200 ml of 20% trichloro-
25 acetic acid, vortexed, incubated at -70ºC for 5 min,
thawed, spun in an Eppendorf centrifuge for 5-10 min at
the maximal speed and 200 ml supernatants were counted in
2 ml Aquasol (NEN) for 10 min.
    Peptide, corresponding to region of p34^{cdc2} undergoing
30 inhibitory tyrosine phosphorylation
(NH2-CKKKVEKIGEGTYGVVYK) (SEQ ID NO. 7) (the peptide
sequence which is additional to cdc2 and added to couple
the peptide to the beads and/or proteins is underlined)
was phosphorylated in vitro using bacterially produced
35 v-Abl (Oncogene Sciences) at conditions described by the

manufacturer and purified on the Seppak column
(Millipore). Final activity incorporated into peptide was
0.7×10^5 cpm/mg. Phosphatase activity of the cdc25 A
protein against peptide (1 mg of peptide were used in each
5   sample) was assayed at the same conditions as for RCML.
Reaction mixture was incubated with acid charcoal as
described (Streuli, M. et al., Natl. Acad. Sci. USA
86:8698-8702 (1989)) and 200 ml from total supernatant of
700 ml were counted as described above.

10   Expression of Cyclin Proteins
       In order to express human cyclins in bacteria
modified pGEX-3X vector (pGEX-Nco) was prepared by
digesting it with SmaI, followed by ligation of the NcoI
linker (described earlier in Experimental procedures);
15   this resulted in a vector where cloning into NcoI site
allowed the proper expression of the foreign cDNA. Human
cyclin BI and A were synthesized by PCR and their sequence
were fully confirmed. cyclin B1 cDNA in the pBluescript
SK(-) was cut with NcoI/SmaI and the resultant 1.3 kb
20   fragment was ligated into pGEX-Nco, digested with EcoRI,
filled in with Klenow fragment and cut with NcoI. The
sequence of cyclin A, including the first ATG codon, was
changed to an ncol site by PCR. To express cyclin A,
plasmids containing the complete open reading frame for
25   cyclin A (p4fl.1) were digested with Ncol and EcoRI and
the resultant 1.4 kb insert was subcloned into pGEX-Nco
cut with Ncol/EcoRI. Human cDNA encoding human cyclin B2
was obtained from Y.Xiong (unpublished), with the first
ATG codon changed by PCR to Ncol site, this cDNA was
30   digested with BamHI, blunt ended with T4 DNA polymerase,
and digested with the Ncol, and the resultant 1.3 kb
fragment was ligated in the pGEX3X-Nco vector prepared as
described above for the ligation of cyclinB1 cDNA. Mouse
CYL1 (cyclin D1) cDNA in the pGEX-3X vector was generous

gift from Dr. C. Sherr. Purification of the expressed
cyclins was performed essentially as described (Smith,
D.B. and K.S. Johnson, Gene 67:31-40 (1988); Solomon, M.J.
et al., Cell 63:1013-1024 (1991)), except that after the
5 first extraction, the cell pellets were resuspended in the
50 mM TrisHCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1%
glycerol, 2M urea and extracted for 10 min on ice. After
centrifugation for 30-60 min at 15000 rpm on the RC-5B
centrifuge (Beckman), the supernatant was filtered through
10 0.22 mm filter (Millipore) and applied on the 2 ml
glutathione-Sepharose column (Pharmacia), equilibrated
with the extraction buffer. columns were washed sub-
sequently with the extraction buffer (10 ml), then with
the same buffer lacking urea (10 ml), and fusion proteins
15 were eluted in the same buffer supplemented with 10 mM
glutathione. Eluted proteins were dialized into
phosphatase assay buffer and concentrated by repeated
dilution-concentration on the Amicon microconcentrators.
Protease inhibitors (PMSF and benzimidine) were added to
20 0.5 and 5 mM subsequently, and the proteins were stored at
4°C for 2-3 days or used immediately on the same day. The
Bradford assay was used to determine protein
concentration.

Microinjection of Antibodies

25   For microinjection experiments HeLa cells were grown
to 20-30 cells in an "island" and injected at time 0 with
affinity purified K144 (1 mg/ml) further depleted on the
#143 peptide conjugated BSA sepharose. The injection was
done in buffer F (20 mM Tris HCl, pH 7.6, 20 mM NaCl, 50
30 mM KCl, 0.5 mM b-mercaptoethanol, 0.1 mM ATP). All cells
in the particular "island" were microinjected and photo-
graphs were taken at 8, 18, 24 and 36 hours after micro-
injection. In a separate set of experiments cells were
photographed at 8, 12, 18 and 24 hours after injection.

Microinjection of the protein A-Sepharose purified rabbit
IgG from the preimmune serum served as a control.

Protein Kinase Assays

For protein kinase assays, p13 beads with bound
5   p34^{cdc2} kinase isolated from the HeLa cells (incubated in
the presence of hydroxyurea (10 mM) for 22 hours followed
by 4 hour release) were washed twice in the buffer
containing 50 mM Tris HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and
incubated for 5 min at 30°C with the additives. Additives
10 included buffer alone, or material eluted with the 0.1 M
glycine/HCl, pH 2.5 from the cdc25 A immunoprecipitates,
done in the presence or absence of 1 mg of an antigenic
peptide (before addition material was neutralized with 1 M
Tris HCl, pH 8.0). The precipitates were washed twice
15 with 50 mM Tris HCl, pH 8.0, 10 mM MgCl$_2$, 1 mM DTT
(PK-buffer), and finally resuspended in 2 volumes of PK
buffer supplemented with 5 mM ATP, 10 mCi of [q^{-32}P] ATP
(3000 Ci/mmol), and 50 mg/ml of histone H1. After
incubation for 15 min at 30°C the reaction was stopped by
20 polyacrylamide gel sample buffer containing SDS. Labeled
proteins were separated on 10% polyacrulamide gels and
detected by autoradiography.

EXAMPLE 1   ISOLATION OF cdc25 A AND cdc B cDNA

A human cdc25 genes has previously been described
25 (Sadhu, K. et al. Proc. Natl Acad USA, 87:5139-5143
(1990)). Further members of what is now shown to be the
human cdc25 family have been isolated by means of a
PCR-based strategy. This strategy made use of three
degenerate oligonucleotide primers designed to correspond
30 to amino-acid regions of consensus between
Drosophila melanogaster string (Edgar, B.A. and P.H.
O'Farrell, Cell 57:177-187 (1989)), S. pombe cdc25

(Russell, P. and P. Nurse, Cell 45:145-153 (1986)) and S. cerevisiae mihl (Russell, P. et al., Cell 57:295-303 (1989)). Amplification of cDNA from a human N-Tera teratocarcinoma library, followed by cloning of the PCR
5 products into a phagemid vector, allowed nucleotide sequencing of the fragments. This established that a cdc25-related fragment different from that previously described had been cloned.

The insert from one PCR-derived clone (p5wl) was used
10 to screen a human cDNA library in the ggt10 vector. From approximately 10^6 plaques screened, nine positive clones were obtained. Eight corresponded to the originally cloned PCR product used as the hybridization probe. This is referred to as cdc25 A. A second cdc25 clone, isolated
15 by using low stringency hybridization with pSwl, was called cdc25 B. The longest cDNA clones of cdc25 A and B were subjected to nucleotide sequencing. The region of each that contains the open reading frame is shown in Figure 1. cdc25 A and cdc25 B are predicted to encode
20 proteins of 526 and 566 amino acids respectively. The calculated isoelectric point for cdc25 A is 6.3, and for cdc25 B is 5.9. Both genes have an initiation codon flanked by a Kozak consensus sequence (PuCC/GATGG) (Kozak, M. Cell 44:283-292 (1986)).
25 Comparison of the amino acid sequence of cdc25 A and cdc25 B and the GenBank data base (release 67) revealed homology to the previously described human cdc25 (Sadhu, K. et al., Proc Natl Acad. Sci.USA 87: 5139-5143 (1990)), referred to herein as cdc25 C. This comparison showed
30 that there is 48% identity in the 273 C-terminal region between cdc25 C and A, and 43% identity between C and B. (Figure 2). Drosophila string shares 34.5% identity to cdc25 A in a 362 amino acid region, and 43.9% identify to cdc25 B in a 269 amino acid region (Figure 2). S.pombe
35 cdc25+ is also related to both cdc25 A and B, though at a

lesser level (Figure 2). Human cdc25 A and cdc25 B
proteins also contain conserved amino acids that
characterize the "cdc25-box", particularly those in the
region potentially involved in cdc25 catalytic activity

5   (L/VFHCEXXXXR) (SEQ ID NO. 8) (Moreno, S. and P. Nurse,
Nature 351:194 (1991); Gautier, J. and J. Maller, EMBO J.
10:177-182 (1991)). All known human cdc25 homologues
contain a stretch of 15 identical amino acids in this
region, called the highly conserved region (SEQ ID NO. 9)

10 (Figure 2). Interestingly, the overall similarity between
different human cdc25 proteins does not greatly exceed
that between humans and such evolutionarily distinct
species as Drosophila.

EXAMPLE 2   Assessment of the Functional Relationship

15   Between Proteins Encoded by Human cdc25 A,
                  cdc25B and Fission Yeast cdc25

To test whether the human cdc25 A and B genes do
indeed encode proteins that are functionally related to
fission yeast cdc25, the human genes were subcloned into

20 the S. pombe autonomously-replicating expression vector,
pARTN (carrying the LEU2 marker under the control of the
constitutive alcohol dehydrogenase promoter, as described
in experimental procedures). After introduction of the
plasmids into an H+ cdc25-22 leul-32 strain, transformants

25 were plated on media either lacking or containing leucine
at a permissive (26°C) or restrictive temperature (36°C).
Both human cDNAs could efficiently rescue the temperature-
sensitive mutation of the cdc25 gene. Cells bearing human
cDNAs were able to form single colonies with a growth rate

30 similar to wild-type cells. Microscopic examination
revealed that cells transformed with either gene were
slightly "wee", a phenotype previously observed in fission
yeast transformed with the wild-type cdc25+ gene on the

same type of vector (Russell, P. and P. Nurse, Cell
45:145-153 (1986)).

EXAMPLE 3   Demonstration That cdc25 A Acts in Mitosis

In order to test the role of cdc25 A, we prepared
5   polyclonal antibodies against a peptide corresponding to
an internal region of the cdc25 A protein (see
Experimental Procedures). This serum was used to
precipitate ^{35}S-methionine labeled HeLa proteins. A
protein of 75kD was specifically precipitated in the
10   absence, but not the presence, of competing antigenic
peptide (data not shown). Stringent detergent conditions
were used that abolish interactions with cdc2 and cyclin.
This molecular weight is higher than predicted from the
amino acid sequence of the gene; however, in vitro
15   translation of the cdc25 A clone also yielded a protein of
75 kD (not shown). To test whether this protein might
activate inactive cyclin B/cdc2, as described in the case
of the Drosophila string protein (Kumagai, A. and W.G.
Dunphy, Cell 64:903-914 (1991)) and also in the case of
20   human cdc25 C (Strausfeld, U. et al., Nature 351:242-245
(1991)), HeLa cell cdc25 A was eluted from an
immunocomplex under conditions of low pH (see Experimental
Procedures). The eluted protein did not possess any
histone kinase activity (data not shown). This protein
25   was mixed with cdc2/cyclin B, prepared by p13-Sepharose
precipitation of an extract of HeLa cells that had been
arrested in hydroxyurea and released for four hours (see
Experimental Procedures). Under these conditions, the
cdc2/cyclin B is relatively inactive as a histone kinase,
30   unless the eluted cdc25 A protein is added (data not
shown).

To address the function of cdc25 A protein in human
cells, affinity-purified anti-peptide antibodies were
microinjected into actively proliferating HeLa cells (see

Experimental Procedures). Islands of injected cells were photographed at 8, 12, 18 and 24 hours, and in another set of experiments at 8, 12, 18, 24 and 36 hours. In some cases, cells were stained with anti-rabbit IgG to confirm

5 the success of the anti-cdc25 antibody microinjection. Analysis of the photographs in three such independent experiments led to the conclusion that the antibodies prevent cells from dividing (Figures 3A, 3B). The percentage of cells in mitosis (defined as rounded-up

10 mitotic figures) increased progressively following microinjection of anti-cdc25A, but not following a control serum (Figure 3A). The cell number in each injected island increased in the case of control serum, but gradually declined in the experimental. This is

15 attributed to the failure of cells to divide, coupled with their eventual death (visualized as shrivelled rounded cells) and their dissociation from the surface of the culture plate. In fission yeast, loss of cdc25 function causes cells to arrest in G2, rather than in mid-mitosis

20 as in the present experiment. This, on the basis of sequence homology, function in fission yeast, and, in the case of cdc25 A, functional studies in human cells, the newly-identified human proteins can be classified as relatives of cdc25.

25 EXAMPLE 4   Activation of cdc25 by B-type Cyclin
          In order to study the regulation of the cdc25 phosphatase activity in vitro, human cdc25 A and B were expressed in bacteria as fusion proteins with gluta-thione-S-transferase (GST, Smith, D.B. and K.S. Johnson,

30 Gene 67:31-40 (1988)). Fusion proteins with a relative molecular weight of 90 kD (cdc25 A) and 88 kD (cdc25 B) were isolated by affinity chromatography on glutathione-Sepharose beads as described (Smith D.B. and K.S. Johnson, Gene 67:31-40 (1988)). Human cyclins A, B1, B2 and murine

D1 (CYLI, Matsushime, H. et al., Cell 65:701-713 (1991))
were expressed as fusion proteins with GST; purified
proteins were obtained by the same method.

To investigate the potential regulation of cdc25
5   activity by cyclin, it was necessary to find a substrate
that bore no conceivable relationship to cdc2, the
presumed physiological substrate of the phosphatase. cdc2
binds to cyclin (Draetta, G. et al., Cell 56:829-838
(1989)) and thus addition of cyclin to a reaction
10   containing cdc2 as the substrate would probably result in
     alteration of the target substrate and confuse the
interpretation of any observed effect. For this reason a
substrate often employed in tyrosine phosphatase studies,
namely reduced, carboxamidomethylated and maleylated
15   lysozyme (RCML) was used. (Tonks, N.K. et al., J. Biol.
Chem. 263:6731-6737 (1988)). This substrate was labelled
on tyrosine residues with ^{32}p and kindly provided by N.
Tonks.

Cyclins purified from bacteria displayed no phospha-
20   tase activity against RCML (Figure 4A). However, cdc25 A
had an endogenous tyrosine phosphatase activity (Figure
4A; see also Experimental Procedures), that is linear for
at least 30 minutes (data not shown). If it is assumed
that all the bacterial cdc25 protein is equally
25   catalytically active, we can calculate that each molecule
of cdc25 releases approximately one phosphate per 10
minutes. Addition of cyclin A or D to the reaction
mixture had neither stimulatory nor inhibitory effect on
the endogenous activity of cdc25 A at any concentration
30   tested (Figure 4A). However, similar addition of either
cyclin B1 or B2 had an approximately four-fold stimulatory
effect (Figure 4A). In the preceding experiments, 10
pmoles of cyclin and cdc25 protein were used in the
reaction mixture. The dependency of the activation of
35   cdc25 on the amount of added cyclin B1 was also

investigated. The assay was performed either without cyclin or with the addition of 1, 2, 5, 10, or 20 pmoles of the cyclin B1. The reaction was performed for 20 min, and terminated by the addition of trichloroacetic acid

5 (TCA). Activation was observed to plateau at 10 pmoles of added cyclin B1 and no further effect was detected at higher concentrations (Figure 5). Thus, under these experimental conditions, maximal activation of cdc25 is achieved by stoichiometric addition of cyclin B.

10   Whether the same stimulatory effect of B-type cyclins on the catalytic activity of cdc25 A could be detected was tested using other substrates including p-nitrophenylphosphate (PNPP), another frequently used PTPase substrate (Tonks, N.K. et al., J. Biol. Chem.

15 263:6731-6737 (1988); Guan, K. et al., Nature 350:359-362 (1991); Dunphy, W.G. and A. Kumagai, Cell 67:189-196 (1991)) and the 18-mer peptide corresponding to the N-terminal region of the cdc2 protein surrounding Tyr15 (see Experimental Procedures). In the first case, the

20 catalytic rate for cdc25 A was activated four to five-fold, specifically in the presence of cyclin B (Figure 4C). 50 pmoles of cyclin and cdc25 protein were used in this PNPP assay. When the 18-mer peptide was used, similar levels of cdc25 A activation by B cyclins

25 were detected (Figure 4B). 10 pmoles of cdc25 protein and cyclin were used in this experiment.

EXAMPLE 5 Cyclin B1/cdc2 Interacts with cdc25A

      To investigate the possibility of stable interaction between cdc25 and cyclin, as predicted from the data on

30 the activation of the cdc25 A phosphatase activity and additional work described in Example 4, immunoprecipitates with the cdc25 A anti-peptide antibody described above were prepared. In this case, immunoprecipitations were performed under conditions favorable for retention of

cdc25 protein complexes (see Experimental Procedures).
Immunoprecipitates were probed with anti-cyclin B1
antibody (kindly provided by J. Pines) or the anti-cdc2
antibody (G6), prepared against C-terminal peptide of the
5   cdc2 (Draetta, G. et al., Nature 336:738-744 (1988)).
Clear signals were detected in both cases, indicating that
human cdc25 protein is present in a complex with both
cyclin B1 and cdc2 (data not shown).

EXAMPLE 6   Selective Inhibition by p13

10   p13 is an essential subunit of the cdc2 protein
kinase. An excess of p13 can, however, inhibit activation
of pre-MPF. To test whether p13 could directly influence
the phosphatase activity of either of the human cdc25
proteins, the phosphatase assay as described in Examples 4
15   and 5 was performed with the addition of a final
concentration of 25 mM, with or without 0.5 mM (10 pmoles)
cyclin B1. In the case of cdc25 A, a 2-3-fold inhibition
of the endogenous phosphatase activity was observed by
adding p13 at 25 mM (Figure 6). This concentration is far
20   higher than that of the cdc25 protein itself (0.3 mM) but
is similar to that required to prevent pre-MPF activation
in vivo or in vitro (Dunphy, W. et al., Cell 54:423-431
(1988); Dunphy, W. and J.W. Newport, Cell 58:181-431
(1989)). Addition of cyclin B1 in an equimolar
25   concentration to the phosphatase was able to substantially
negate the inhibitory effect of p13, causing an eight-fold
activation (Figure 6). The behavior of cdc25 B was quite
different. In preliminary experiments, it was found that
the pH optimum for this phosphatase is 8.8 (as opposed to
30   8.0 for cdc25 A). At this pH, cyclin B1 could activate
cdc25 B to a similar degree to cdc25 A. However, no
effect of p13 on the activity of cdc25 B was observed
either in the presence or absence of cyclin B (Figure 6).

EXPERIMENTAL PROCEDURES

The following experimental procedures were used in the work described in Examples 7-13.

Oocyte and Extract Preparation

5   Xenopus laevis propbase oocytes were prepared as described (Jessus, C. et al., FEBS Letters 266: 4-8 (1987)) and were induced to mature by 1 mM progesterone. Xenopus metaphase unfertilized eggs were activated in 1 mM HEPES pH7.4, 8.8 mH NaCl, 10 mg CaCl$_2$, 33 mH Ca(NO$_3$)$_2$, 0.1
10   mH KCl, 82 mM MgSO$_4$, 5 mg/ml Ca^{2+}-ionophore A-23187 (Sigma) and 100 mg/ml cycloheximide (Sigma). After 40 min, eggs were either homogenized and referred as "activated eggs", or washed, transferred to incubation buffer (Jessus, C. et al., FEBS Letters 266:4-8 (1987)) and homogenized at
15   different times. To prepare extracts, oocytes were washed extensively in extraction buffer EB (Cyert, H.S. and M.W. Kirschner, Cell 53:185-195 (1988)) 80 mM b-glycerophosphate pH7.3, 20 mM EGTA, 15 mM MgCl$_2$, 1mM DTT), then lysed at 4ºC in one volume of EB with protease
20   inhibitors (25 mg/ml leupeptin, 25 mg/ml aprotinin, 1 mM benzamidine, 10 mg/ml pepstatin, 10 mg/ml soybean trypsin inhibitor and 1 mM PMSF) and centrifuged for 1 h at 100,000xg at 4ºC. The supernatant was then filtered through 0.22 mm Millex-GV filters (Millipore) before use.

25   Prpearation and Use of p13-Sepharose Beads

P13 was purified and conjugated to sepharose as previously described (Brizuela, L. et al., EMBO J. 6:3507-3514 (1987)). After preincubation for 1 h with Sepharose CL-6B and centrifugation to remove non-specific
30   binding, 100 ml of oocyte extracts were incubated for 90 rain at 4ºC under constant rotation with 400 ml of EB plus protease inhibitors and 20 ml of p13-Sepharose beads. p13-Sepharose beads were further washed three times in EB,

then either resuspended in 80 ml of Laemmli sample buffer
(Laemmli, U.K., Nature 227:680-685 (1970)) and boiled for
3 min, or immediately used for histone HI kinase assay.

Preparation of 0-33% Ammonium Sulfate Extracts

5   Prophase oocytes were rinsed extensively in EB, then
lysed in one volume of EB with protease inhibitors at 4°C
and centrifuged at 41,000 rpm for 90 rain at 4°C in Ti.41
rotor (Beckman). The supernatant was removed and filtered
through 0.22 mm Millex-GV filters (Millipore). Ammonium
10 sulfate fractionation was carried out by addition of 0.5
volume of a saturated solution of ammonium sulfate in EB
to the extract, incubation on ice for 45 min,
centrifugation at 41,000 rpm for 90 min at 4°C and
resuspension of the pellet in one-tenth of the initial
15 volume to a final protein concentration of 15 mg/ml, as
determined with the BioRad protein assay kit with
q-globulin as the standard. This extract (termed 0-33%
fraction) was dialyzed for 2 h at 4°C against EB in the
presence of protease inhibitors and stored at -70°C until
20 use. For activation, extracts were incubated at room
temperature with 1 mM ATP, 50 mg/ml creatine phosphokinase
(Boehringer Mannheim) and 10 mM creatine phosphate
(Boehringer Mannheim).

Antibodies

25   Fission yeast cdc25 protein was produced in
Escherischia coli expressing the full-length protein
(Ducommun, B. et al., Biochem. Biophys. Res. Comm.
167:301-309 (1990)). Bacterially produced cdc25 protein
was purified and solubilized as described by Kumagai and
30 Dunphy (Kumagai, A. and W.G. Dunphy, Cell 64:903-914
(1991)). To purify B1 anti-cdc25 serum (Ducommun, B. et
al., Biochem. Biophys. Res. Comm. 167:301-309 (1990)),
bacterially expressed cdc25 protein was subjected to

SDS-polyacrylamide electrophoresis and extracted by
incubation of the excised gel pieces in PBS (phosphate
saline buffer) (0.1% SDS 0.5% b-mercaptoethanol) at 37ºC
for 16 h. After centrifugation, the protein was

5 concentrated on Centricon-10 microconcentrators (Amicon)
and incubated with nitrocellulose (0.45 mM; Schleicher and
Schuell) for 3 h at room temperature. After three ten
minute washes in PBS (0.1% SDS), filters were blocked for
4 h at room temperature with PBS containing 1.5% BSA

10 (bovine serum albumin, Boehringer Mannheim) and 0.5%
Tween-20. After three ten-minute washes in PBS (0.1%
SDS), filters were incubated at room temperature for 16 h
with B1 anti-cdc25 serum (Ducommun, B. et al. Biophys.
Res. Comm. 167:301-309 (1990)), and diluted four times in

15 PBS 1.5% BSA. Filters were then washed three times for 10
rain with PBS (0.1% Tween-20) and once for 10 min with
PBS. Purified anti-cdc25 antibody was eluted with 1 ml of
100 mM glycine pH2.5, and 200 ml of 1 M TRIS pH8.0 was
added after 1 min. After addition of 300 ml of PBS (10%

20 BSA, 0.5% NaN$_3$), the purified antibody was stored at 4ºC
until use. For some control experiments, the purified
antibody was preadsorbed overnight at 4ºC with 10 mg/ml
purified bacterially expressed yeast cdc25 protein before
Western blotting.

25 Anti-B2 cyclin antibody was a gift from J. Gautier
(rabbit polyclonal purified antibody directed against
Xenopus cyclin B2; Gautier, J. et al., Cell 60:487-494
(1990); Gautier, J. and J. Maller, EMBO J. 10:177-182
(1991)). Anti-cdc2 antibody was a rabbit polyclonal

30 purified antibody directed against thr full-length
Schizosaccharomyces pombe cdc2 (Draetta G. et al., Cell
50:319-325 (1987)). Anti-phosphotyrosine antibody was a
mouse IgG monoclonal antibody (Ab-1, Oncogene Science).
The sensitivity of this anti-phosphotyrosine antibody

35 ought to have been be sufficient to allow the detection of

phosphotyrosine in the cdc25-associated cdc2, since a comparable amount of prophase cdc2 was easily recognized. Therefore, the absence of signal observed in metaphase cdc2 bound to cdc25 suggested that this population of cdc2
5   was not phosphorylated on tyrosine.

Immunoprecipitation and Western Blot Analysis

100 ml of oocyte extracts in EB were mixed with 400 ml of Eb and incubated for 1 h at 4°C with 30 ml of protein A-agarose beads (Pierce). Anti-cdc25 antibody
10   (dilution 1:100), anti-cyclin B2 antibody (dilution 1:50) or anti-cdc2 antibody (dilution 1:500) were then added to the supernatant and after a 5h incubation at 4°C, 30 ml of protein A-agarose beads were added. After an additional 1 h incubation at 4°C, the beads were either washed four
15   times in EB and then eluted by boiling for 30 min in 80 ml Laemmli sample buffer or resuspended in kinase buffer (50 mM TRIS pH7.4, 10 mM MgCl$_2$, 5 mM EGTA, 1 mM DTT) for a subsequent histone HI kinase assay.

To elute Xenopus cdc25 protein from immunoprecipi-
20   tates, immunocomplexes were resuspended in 250 ml of 100 mM glycine pH2.5. After a 2 min stirring, 50 ml of 1 M TRIS pH8.0 was added. The supernatant was recovered, concentrated on Centricon-10 microconcentrators (Amicon) and bovine serum albumine was added to a final concen-
25   tration of 0.1%.

Electrophoresis and Western blot analysis with anti-cdc25 antibody (dilution 1:500), anti-cyclin B2 antibody (dilution 1:100) or anti-cdc2 antibody (dilution 1:000) were performed as previously described (Booher,
30   R.N. et al., Cell 58:584-497 (1989)). By scanning immunoblots of initial extracts before anti-cdc25 immunoprecipitation, extracts after anti-cdc25 immunoprecipitation and anti-cdc25 immunoprecipitates

(FujiX Bas 2000 Image Analyzer), it was we estimated that 70% of the full cellular amount of cdc25 was immunoprecipitated by the anti-cdc25 antibody. In a parallel way, the amount of p72 associated with cdc2 or cyclin B2 in

5 immunoprecipitates was quantified by Image Analyzer (FujiX Bas 2000), by using anti-cdc25 immunoblots of crude extracts as a reference of the full cellular amount of cdc25. 20% of the total cellular amount of cdc25 was found either in anti-cdc2 immunoprecipitates or in

10 anti-cyclin B2 immunoprecipitates. To quantify the amount of cdc2 or cyclin B2 associated with cdc25, equal amounts of oocyte extracts (from 10 oocytes, equivalent to 200 mg of proteins) were either precipitated on p13-Sepharose or immunoprecipitated with anti-cdc25 antibody. p13-sepha-

15 rose beads completely clear the extract of cdc2 and cyclin B2 as ascertained by Western blotting (data not shown) and, therefore, p13-precipitate represents the full cellular amount of cdc2 and cyclin B2. On the other hand, the anti-cdc25 immunoprecipitate contains only the cdc2

20 and the cyclin B2 that are associated with p72. Both p13-precipitates and anti-cdc25 immunoprecipitates (each the equivalent of 10 oocytes) were loaded on the same electrophoresis gel and blotted with the anti-cdc2 antibody or the anti-cyclin B2 antibody. The relative

25 amounts of cdc2 and cyclin B2 detected in both extracts were determined by PhosphorImager (Molecular Dynamics) or Image Analyzer (FujiX Bas 2000). The amount of cdc2 present in p13-Sepharose precipitate is 20-fold higher than that detected in the anti-cdc25 immunoprecipitate.

30 Thus, 5% of the total cdc2 is associated with p72. The amount of cyclin B2 present in p13-Sepharose precipitates is 6-fold higher than that detected in the anti-cdc25 immunoprecipitate. Thus, 17% of the total cyclin B2 is associated with p72.

Histone H1 Kinase Assay

p13-precipitates or immunocomplexes were washed three times in kinase buffer and then resuspended in 50 ml of kinase buffer containing 0.2 mg/ml histone H1 (Boehringer

5 Mannheim), 50 mM ATP and 1 mCi[q^{32}P]ATP (PB.10168, Amersham). After a 30 min incubation at 30°C, the reactions were terminated by the addition of 30 ml Laemmli sample buffer (Laemmli, U.K., Nature 227:680-685 (1970)). Samples were electrophoresed on a 12% polyacrylamide gel.

10 After staining with coomassie blue and autoradiography, ^{32}P incorporation into histone H1 was quantified by scintillation counting of excised gel pieces.

Protein samples from the 0-33% fraction (in a volume of 10 ml of EB) were mixed on ice with 40 ml of kinase

15 buffer containing 0.2 mg/ml histone H1, 25 mM ATP, 2 m Ci[q^{32}P]ATP and 10 mM cAMP dependent protein kinase inhibitor peptide (P3294, Sigma). After incubation for 10 min at 30°C, samples were treated as previously described.

EXAMPLE 7   cdc25 Protein in Xenopus Oocytes

20 An anti-cdc25 serum directed against fission yeast cdc25 was used to determine whether a cdc25 protein is present in Xenopus oocytes. This serum, previously referred to as B1 (Ducommun, B. et al., Biochem. Biophys. Res. Comm. 167:301-309 (1990)), was affinity purified as

25 described in the Experimental Procedures. It recognizes the full-length yeast cdc25 product expressed in E. coli but no signal is detectable in an E. coli lysate before transcriptional cdc25 induction of cdc25 (Ducommun, B. et al., Biochem. Biophys. Res. Comm. 167:301-309 (1990)).

30 Extracts were prepared from the following cells: meiotic prophase-blocked oocytes; meiotic metaphase unfertilized eggs; eggs activated in the presence of cycloheximide, that therefore lack cyclin and are blocked in an interphase state (Murray, A.W. and Kirschner, M.

<u>Nature</u> <u>339</u>:275-280 (1989)); and eggs after 120 min of
activation (after completion of the first MPF cycle).
These extracts were probed with the affinity-purified
serum in an immunoblot. A 72 kD polypeptide was detected
5 in each sample. No signal was detected using the same
procedure but substituting preimmune serum or purified
antibody preadsorbed with soluble bacterially-expressed
yeast cdc25 protein for the affinity-purified serum (data
not shown). Furthermore, two other purified polyclonal
10 antibodies directed against the yeast cdc25 protein were
able to recognize the same 72 kD protein from Xenopus
extracts. (Ducommun, B. <u>et al</u>., <u>Biochem. Biophys. Res.</u>
<u>Comm.</u> <u>167</u>:301-309 (1990)).

To test whether the 72 kD species might be immuno-
15 precipitated by the anti-cdc25 antibody, extracts from
prophase oocytes, metaphase unfertlllzed eggs and inter-
phase eggs activated in the presence of cycloheximide were
precipitated with the purified anti-cdc25 antibody and
probed with the same purified serum in immunoblots. Again,
20 a protein of 72 kD was speclfically detected by the cdc25
antibody (data not shown). In contrast, no signal was
detected when the same procedure was used in the absence
of Xenopus extract, formally demonstrating that the 72 kD
protein observed in the immunoprecipitates is not due to
25 the presence of cdc25 protein in the antibody preparation
(a contamination that could occur during immuno-affinity
purification of the antibody).

To obtain soluble 72 kD polypeptide, proteins were
eluted from anti-cdc25 immunoprecipitates at low pH (see
30 Experimental Procedures) and the amount of 72kD protein
was determined by immunoblotting with the cdc25 antibody.
Again, the same level of 72 kD protein was found in
prophase oocytes, metaphase unfertillzed eggs, inter-
phase-blocked activated eggs and eggs after the completion
35 of the first MPF cycle (data not shown).

EXAMPLE 8   Demonstration That cdc25 Activates the M-
              phase Kinase

Human and Drosophilia cdc25 proteins are able to
trigger activation of cdc2/cyclin B in vitro (Kumagai, A.
5 and W.G. Dunphy, Cell 64:903-914 (1991); Strausfeld, U. et
al., Nature 351:242-245 (1991)) by dephosphorylating cdc2
(Dunphy, W.G. and A. Kumagai, Cell 67:189-196 (1991);
Gautier, J. et al., Cell 67:197-211 (1991)). As a further
test that the anti-cdc25 antibody recognized Xenopus
10 cdc25, it was investigated whether the 72 kD protein
eluted from immunocomplexes could stimulate inactive cdc2.
To prepare inactive enzyme from prophase oocytes   ,
p13-Sepharose beads were used. Xenopus cdc2 protein binds
strongly and quantitatively to fission yeast p13. (Dunphy,
15 W. et al., Cell 54:423-431 (1988)). The p13-Sepharose
bound cyclin B/cdc2 complex from prophase oocytes has a
low histone H1 kinase activity. Protein eluted from
anti-cdc25 immunoprecipitates of either prophase oocytes
or metaphase unfertilized eggs was added to inactive
20 prophase p13-bound cdc2. After a 30 min preincubation at
30°C in the presence of cdc25-immunocomplex eluates, the
p13-precipitate was extensively washed and then assayed
for histone H1 kinase activity. Both prophase and
metaphase cdc25 stimulated histone H1 kinase activity
25 12-fold. The possibility that some of the histone HI
kinase activity present in the anti-cdc25 immunocomplexes
(see below) might be responsible for this increase of
kinase activity was eliminated. First, the p13-Sepharose
precipitate was extensively washed after preincubation
30 with the immunoeluted material, and before assay of kinase
activity. Second, the histone H1 kinase activity found
associated with the eluted metaphase proteins was
insufficient to account for the observed 12-fold
stimulation of the p13-bound enzyme (approximately 500
35 units of final activity). Third, the prophase

immuno-eluted material was also able to activate cdc2, although it did not contain any kinase activity (data not shown). It was therefore concluded that an active Xenopus cdc25 protein was precipitated by the affinity-purified
5 anti-cdc25 antibody from both prophase oocytes and metaphase eggs. It is surprising that active p72 could be extracted from Xenopus oocytes in which cdc2/cyclin B is inactive and tyrosine phosphorylated.

It was also tested whether p72 from either prophase
10 oocytes or metaphase unfertilized eggs could affect the activity of either fully activated cdc2/cyclin from metaphase unfertilized eggs or cdc2 that is inactive in the absence of cyclin (material extracted from eggs activated in the presence of cycloheximide). In neither
15 case did p72 have any effect on the histone H1 kinase activity of cdc2 (data not shown). The 135 units of activity found in one sample of activated eggs is probably due to the basal activity of cdc2 from activated eggs (66 units) combined with the kinase activity associated with
20 metaphase cdc25 and therefore does not represent a real stimulation of cdc2. It was concluded that p72 only acts on the tyrosine phosphorylated enzyme.

Example 9   Demonstration That Activation of pre-MPF
             Requires cdc25
25   Xenopus prophase oocytes contain an inactive form of MPF that can be activated by a post-translational mechanism both in vivo (Wasserman, W. and Y. Masui, Exp. Cell. Res. 91:381-388 (197S); Gerhart, J. et al., J. Cell Biol. 98:1247-1255 (1984)) and in vitro (Cyert, M.S. and
30 M.W. Kirschner, Cell 53: 185-195 (1988); Dunphy, W.G. and J.W. Newport, Cell 58: 181-191 (1989)). Addition of an ATP-regenerating system to a prophase oocyte extract (33% ammonium sulfate precipitated fraction) is sufficient to induce tyrosine dephosphorylation of cdc2 and stimulation

of its latent activity (Cyert, M.S. and M.W. Kirschner,
Cell 53: 185-195 (1988); Dunphy, W.G. and J.P. Newport,
Cell 58: 181-191 (1989)). In order to determine if
endogenous p72 was required for this activation process,
5   the effect of adding anti-cdc25 antibody to the 0-33%
ammonium sulfate fraction from phophase oosytes was
explored. 200 ml of the 0-33% ammonium sulfate fraction
of high speed extract of phophase oocytes was incubed for
15 min at 40°C. At 0 min, samples were transferred to
10   room temperature, and 1 mM ATP, 10 mM creative phosphase
and 50 mg/ml creative phosphokinase were added.
Following the addition of this ATP-regenerating system to
the extract, the histone H1 kinase was rapidly activated
(Fig. 8). By contrast, a 15 min preincubation of the
15   extract with anti-cdc25 antibody resulted in a prolonged
inhibition of the activation process. Addition of the
preimmune anti-cdc25 serum had no effect (Fig. 8). This
result suggests that the endogenous p72 is required for
histone H1 kinase activation and is inactivated after
20   immunocomplexing with the antibody. It was further found
that bacterially-expressed cdc25 protein at 100 mg/ml,
when added at 60 minutes, can overcome the inhibition
caused by the anti-cdc25 antibody (Fig. 8), indicating
that the antibody acts specifically on the endogenous
25   cdc25 protein.

EXAMPLE 10   Demonstration of an Association Between
              cdc25 and cdc2 at M-phase
       To investigate further the mechanism of cdc2 acti-
vation by cdc25, the possibility that cdc25 might directly
30   associate with the M-phase enzyme was tested. Extracts of
either prophase oocytes, metaphase unfertilized eggs or
activated eggs were immunoprecipitated with an anti-cdc2
antibody and probed with the same anti-cdc2 antibody. As
expected, a strong signal was obtained (data not shown).

Since the anti-cdc2 antibody recognized a single 34 kD band, it was assumed that this antibody does not react with cdk2, a 32 kD cdc2-like protein encoded by the Xenopus Egl gene (Paris, J. et al., Proc. Natl. Acad. Sci.

5 USA 88:1039-1043 (1991)). Similar anti-cdc2 immunoprecipitates were probed with the purified anti-cdc25 antibody. A 72 kd band was observed in the metaphase unfertilized eggs, but not in the resting prophase oocytes or in the eggs activated in the presence

10 of cycloheximide. In a control experiment in which the purified anti-cdc25 antibody was preadsorbed with bacterially expressed cdc25 protein before immunoblotting, no signal was detected. These results indicate that cdc25 stably associates with cdc2 at M-phase.

15 To further test the existence of an association between cdc2 and cdc25 the converse experiment was also performed. Cdc25 was immunoprecipitated from prophase oocytes, metaphase unfertilized eggs and activated eggs using the purified anti-cdc25 antibody. An equal amount

20 of cdc25 was precipitated in each case (data not shown). The anti-cdc25 immunoprecipitates were then probed with the anti-cdc2 antibody. A 34 kD protein was detected in the metaphase unfertilized eggs, but not in the prophase oocytes or in the activated eggs (data not shown). To

25 confirm that the 34 kD protein detected in this experiment was indeed cdc2, prophase oocyte, metaphase unfertilized egg and activated egg extracts were first depleted of the cdc2/cyclin B complex by preincubation with p13-Sepharose and then immunoprecipitated with the purified anti-cdc25

30 antibody. Immunoblotting these immunocomplexes with anti-cdc2 antibody revealed complete depletion of the 24 kD protein (data not shown). Therefore, it was concluded that the 34 kD protein was cdc2. Moreover, cdc2, which is present at the same level in prophase oocytes, metaphase

35 eggs and interphase eggs, was not recognized in an

immunoblot by the purified anti-cdc25 antibody, indicating
that there was no cross-reactivity between cdc2 and the
anti-cdc25 antibody. By quantifying the signal in
immunoblots (see Experimental Procedures), it was
5 estimated that the amount of cdc2 present in anti-cdc25
immunoprecipitates represented approximately 5% of the
total cellular cdc2 at metaphase and that the amount of
cdc25 present in anti-cdc2 immunoprecipitates represented
20% of the cellular content of cdc25.

10 EXAMPLE 11   Demonstration That Cyclin B is Associated
              with cdc2 and cdc25 at M-Phase
        Since the active cdc2 from M-phase is associated with
   cyclin (Brizuela, L. et al.., Proc. Natl. Acad. Sci. USA
   86:4362-4366 (1989); Draetta, G. et al., Cell
15 56:829-838 (1989); Gautier, J. et al., Cell 60:487-494
   (1990)), it was further investigated whether cyclin B is
   present in association with cdc2 and cdc25 at M-phase.
   Extracts of either prophase oocytes, metaphase
   unfertilized eggs or activated eggs were precipitated with
20 p13-Sepharose and probed with an anti-cyclin B2 antibody.
   Cyclin B2 was present in both prophase oocytes and
   metaphase unfertilized eggs (data not shown). As already
   noted (Gautier, J. and J. Maller, EMBO J. 10:177-182
   (1991); Kobayashi, A.H. et al., J. Cell Biol. 114:755-765
25 (1991)), two immunoreactive bands of cyclin B2 are
   detectable, of which the upper band was a phosphorylated
   form appearing during meiotic maturation. In contrast,
   cyclin B2 was not detectable in eggs activated in the
   presence of cycloheximide (data not shown). The same
30 extracts were immunoprecipitated with the anti-cyclin B2
   antibody and then probed with the purified anti-cdc25
   antibody. The 72 kD protein was detected in association
   with cyclin B2 in the metaphase eggs but not in the
   prophase oocytes or in the interphase eggs (data not

shown). The converse experiment was then performed. The three types of cell extracts were immunoprecipitated with the purified anti-cdc25 antibody and probed with the anti-cyclin B2 antibody. Cyclin B2 was associated with

5 cdc25 in metaphase unfertilized eggs, but not in resting prophase oocytes or activated eggs (data not shown). The phosphorylated form of cyclin B2 was predominantly associated with cdc25. As a control experiment, prophase oocyte, metaphase egg and activated egg extracts were

10 first depleted of cdc2/cyclin B by incubation with p13-Sepharose and then immunoprecipitated with the anti-cdc25 antibody. No signal was detected after probing these extracts with the anti-cyclin B2 antibody, indicating that the 51 kD band previously detected was

15 indeed cyclin (data not shown). It was therefore concluded that cdc25 binds to the cyclin B/cdc2 complex at metaphase. The amount of cdc25 present in anti-cyclin B2 immunoprecipitates was estimated to be the same as the proportion of cdc25 previously found in association with

20 cdc2 (20% of the full cellular content of cdc25). In contrast, it was determined that cdc25-associated cyclin B2 represents 17% of the total population of cyclin B2, which is a higher percentage than the amount of cdc25-associated cdc2 (5%).

25 EXAMPLE 12   M-phase Kinase Associated with cdc25 is
              Active
    At metaphase, cdc2 is predominantly tyrosine dephos-phorylated and active as a histone H1 kinase. Since cdc2 is associated with cdc25 only at metaphase, the tyrosine

30 phosphorylation state and the kinase activity of the complexed cdc2 were investigated. By immunoblotting p13-Sepharose precipitates with an anti-phosphotyrosine antibody, it was confirmed that cdc2 was heavily tyrosine phosphorylated in prophase oocytes and substantially

dephosphorylated in metaphase unfertilized eggs, although different batches of metaphase eggs display a somewhat different degree of cdc2 tyrosine dephosphorylation, as previously demonstrated (Dunphy, W.G. and J.W. Newport,

5 Cell 58:181-431 (1989); Jessus, C. et al., FEBS Letters 266:4-8 (1990). No tyrosine phosphorylation of cdc2 could be detected in eggs that were activated in the presence of cycloheximide and thus lack cyclin B. (See also Solomon, M.J. et al., Cell 63:1013-1024 (1991)). When anti-cdc25

10 immunocomplexes from prophase oocytes, metaphase unfertilized eggs or activated eggs were probed with the same anti-phosphotyrosine antibody, no phosphotyrosine-containing proteins were detected, despite the presence of abundant cdc2 in the immunocomplex from metaphase

15 unfertilized eggs (data not shown). If the cdc25-associated cdc2 were substantially tyrosine phosphoryl-ated, a signal of sufficient strength would have developed in the immunoblot. This result suggested that the fraction of cdc2 associated with cdc25 in metaphase

20 unfertilized eggs was likely to be active as a histone H1 kinase. This was found to be true: the kinase activity in p13-Sepharose precipitates was very low in prophase oocytes, was increased 31-fold in metaphase unfertilized eggs and declined during activation in the presence of

25 cycloheximide. Histone H1 kinase activity was detected in anti-cdc25 immunoprecipitates from metaphase eggs. The activity detected in anti-cdc25 immunoprecipitates from prophase oocytes and activated eggs was comparable to the background levels (data not shown), indicating that no

30 cdc2 kinase was present in these extracts. By comparing the relative metaphase kinase activity in P-13 Sepharose precipitates and anti-cdc25 immunoprecipitates (approximately 20-fold different) it was found that the specific activity of cdc2 was essentially identical in

35 each sample.

EXAMPLE 13   Association Between cdc2/cyclin B and
                cdc25 is Periodic

The abundance of the Xenopus cdc25 protein appears
not to vary during meiotic maturation or in the first
5 embryonic cycle (data not shown). However, the protein
was only found in association with cdc2 and cyclin B in
metaphase unfertilized eggs. To investigate this more
closely, metaphase unfertilized eggs were parthenogene-
tically activated in the presence of CA^{2+}-ionophore and
10 calcium, and histone H1 kinase activity was assessed in
p13-Sepharose precipitates during the first 150 min. At
various intervals, 100 eggs were homogenized, centrifuged,
and precipitated. The histone H1 kinase activity
disappeared about 20 min after activation, reappeared
15 between 60 and 90 min at time of the first cleavage,
declined again and finally peaked at time of the second
mitotic cleavage (Fig. 9). Samples taken from the same
cell extracts were immunoprecipitated with anti-cdc25
antibody and immunoblotted with anti-cdc2 serum to
20 estimate the extent of association. Relative amounts of
cdc2 present in the anti-cdc25 immunoprecipitates were
quantified by Phosphor-Imager. The periodic interval of
the association between cdc2/cyclin B complex and cdc25
was identical to the periodicity of the p13-bound enzyme
25 activity (Fig. 9). However, a slight phase shift was
noted. The association peaked slightly ahead of the
overall histone H1 kinase. In repeated experiments (data
not shown), the pattern of association was always the
same. However, in some cases the phase shift between the
30 histone H1 kinase activity and the association between
cdc2/cyclin B and cdc25 was less obvious.

Experimental Procedures

The following materials, methods and procedures were
used in carrying out the work described in Examples 14-18.

Materials and Methods

Chemicals sodium fluoride, sodium orthovanadate, nitrophenol, cis-platinum, isopropyl β-D-thiogalacto-pyranoside (IPTG), 1-methyladenine, dithiothreitol (DDT),
5 EGTA, EDTA, MOPS, β-glycerophosphate, leupeptin, aprotinin, soybean trypsin inhibitor, benzamidine, histone H1 (type III-S), CNBr-activated sepharose 4B, glutathione-agarose (G 4510), glutathione (G 4251), nonidet P40 (NP40), Tris, LB Broth base, were obtained from
10 Boehringer-Mannheim; p-nitrophenylphosphate (p-NPP) (disodium salt hexahydrate, ref. 12.886.82) was from Janssen Chimica.

$[\gamma^{-32}P]$-ATP (PB 168) and $^{125}I]$-protein A (IM 144) were obtained from Amersham.
15 G1 anti-p34^{cdc2} antibodies and anti-p80^{cdc25} antibodies (directed against the cdc25C phosphatase peptide H$_2$N-QEGERQLREQIALLVKDMS-COOH) were kindly provided by Dr. G. Draetta (Heidelberg); anti-cyclin B^{cdc13} (starfish) antibodies were generously donated by Dr. T. Kishimoto
20 (Tokyo); anti-phosphotyrosine antibodies were generously given by Dr. J.Y.J. Wang (La Jolla); antibodies against H$_2$N-VEKIGEG<u>TY</u>GVVYKARHKLS-COOH (a p34^{cdc2} peptide containing the regulatory threonine-14 and tyrosine-15 residues) were kindly provided by Dr. L. Tung (Philadelphia). This last
25 antibody does not recognize tyrosine-phosphorylated p34^{cdc2} but only tyrosinedephosphorylated p34^{cdc2} but only tyrosinedephosphorylated p34^{cdc2}.

Buffers

Oocyte homogenization buffer contained 60 mM β-
30 glycerophosphate, 15 mM p-NPP, 20 mM MOPS pH 7.2, 15 mM EGTA, 15 mM MgCl$_2$, 1 mM DTT, 0.1 mM sodium vanadate, 0.1 mM sodium fluoride, 10 μg leupeptin/ml, 10 μg aprotinin/ml, 10 μg soybean trypsin inhibitor/ml, 100 μM benzamidine. This buffer had previously been shown to

stabilize the starfish meiotic oocyte M phase-specific histone H1 kinase (Pelech, S.L. et al., Biochemistry 26:7960-7968 (1987)).

5   Bead buffer contained 50 mM Tris pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP40, 10 µg leupeptin/ml, 10 µg aprotinin/ml, 10 µg soybean trypsin inhibitor/ml and 100 µM benzamidine.

Tris-Buffered Saline (TBS) contained 50 mM Tris pH 7.4, 150 mM NaCl.

10   Phosphate-Buffered Saline (PBS) contained 9.6 mM phosphate, 2.7 mM KCl, 140 mM NaCl.

Lysis buffer contained 1% NP40, 1 mM EDTA, 1 mM DTT, 10 µg leupeptin/ml, 10 µg aprotinin/ml, 10 µg soybean trypsin inhibitor/ml and 100 µM benzamidine/ml in PBS.

15   Tris buffer A contained 50 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT.

Elution buffer contained 10 mM glutathione in Tris buffer A.

Preparation of G2 and M Phase Oocytes

20   G2 and M phase oocytes were prepared as follows: gonads were removed from mature starfish (Marthasterias glacialis), collected in Northern Brittany. They were either directly frozen in liquid nitrogen and kept at -80°C (G2 oocytes) or incubated with 10 µM 1-methyladenine

25   in natural seawater for 10 min (M. oocytes). By that time all the oocytes had entered the M phase, although they were still in the gonads. These were then removed from the incubation medium, rapidly blotted on filter paper, directly frozen in liquid nitrogen and kept at -80°C.

30   Transfer buffer contained 39 mM glycine, 48 mM Tris, 0.37% SDS, 20% methanol.

Bacterial Growth and cdc25A Induction

An E. coli strain (BL 21(DE3)) containing a plasmid
encoding the genes fusion construct of glutathione-S-
transferase (GST) and human cdc25A under the control of
5   IPTG was used (Galaktinonov, K. and D. Beach, Cell
67:1181-1194 (1991)). E. coli were first grown overnight
at 37°C in the presence of 100 μg ampicillin/ml LB medium.
Four ml of this preculture were inoculated/liter of LB
containing 100 μg ampicillin/ml. Incubation was pursued
10   at 30°C until the culture O.D. at 500 nm had reached a
value between 0.8 and 1.00 (about 4-5 hrs). At this
moment, 0.4 mM IPTG was added and the culture incubated at
25°C for at least 7 hours. Cells were then harvested by a
3000 g centrifugation for 15 min at 4°C. Pellets were
15   kept frozen at -80°C until extraction.

Example 14 $p80^{cdc25}$ Controls $p34^{cdc2}$/cyclin B Activation

Inactive pre-MPF (G2) is constituted of cyclin B and
$p34^{cdc2}$ phosphorylated on its threonine-14 and tyrosine-15
residues. $p80^{cdc25}$ is the phosphatase which dephosphorylats
20   the tyrosine-15 residue, and possibly threonine-14. Its
action leads to activation of the $p34^{cdc2}$/cyclin B^{cdc13}
kinase responsible for induction of the G2/M transition.
The interaction of these components and activation of
inactive pre-MPF (G2) is represented in Figure 10. An
25   agent to be tested for its ability to alter stimulation of
kinase activity is combined with the inactive pre-MPF (G2)
and the effects, if any, are determined. If an agent
tested is an inhibitor, the inactive pre-MPF will not be
activated.

30   Example 15   Production and Purification of GST cdc25A
Phosphatase

A fusion construct between the glutathione-S-
transferase (GST) gene and human cdc25A was built in a

plasmid vector (Galaktionov, K. and D. Beach, Cell
67:1181-1194 (1991)). Transfected and expressed in E.
coli, it produced large amounts of the corresponding
fusion protein which was purified by affinity
5 chromatography on glutathione-agarose beads. The
protocols of production, purification and assay of the
GST-cdc25A phosphatase are described in detail below.
Production involved culture of recombinant E. coli and
classical induction of GST-cdc25A expression by IPTG.
10 One-step affinity-chromatography on glutathione-agarose
allowed the purification of the GST-cdc25A phosphatase.
The optimum ratio of bacterial extract volume/glutathione-
agarose volume was found to be 6-10 to 1. GST-cdc25A was
either preserved as the bacterial pellet (very stable),
15 the supernatant of the centrifuged bacterial extract or
after affinity-purification ad in the presence of 40%
glycerol (final volume).

The bacterial pellet was disrupted by sonication in
lysis buffer at 4°C. The homogenate was centrifuged for
20 30 min at 4°C at 100,000 g; the supernatant was
recentrifuged under similar conditions; the final
supernatant was then immediately mixed and rotated with
glutathione-agarose beads (equilibrated with lysis buffer)
for 30 min at 4°C (6-10 volumes of supernatant/1 volume of
25 packed beads). The glutathione-agarose beads were washed
three times with 10 volumes of lysis buffer, followed by
four washes with 10 volumes of Tris buffer A. Elution of
the fusion protein was induced by 3-4 successive washes
with 10 mM glutathione in Tris buffer A. The efficiency
30 of the elution was monitored by a phosphatase assay.
Active fractions were pooled and used directly or
supplemented with 40% glycerol prior to storage at -80°C.

Glutathione-agarose beads easily recycled by a wash
with 1 M NaCl, followed by equilibration with lysis
35 buffer.

Example 16   Assay of the GST-cdc25A Phosphatase Activity
Towards p-Nitrophenylphosphate

GST-cdc25A phosphatase activity can be very conveniently assayed using the chromogenic substrate

5   p-nitrophenylphosphate (p-NPP). Optimal conditions for several parameters were determined with a one ml assay, as described below. Results are represented graphically in the figures: amount of GST-cdc25A phosphatase (Figure 12A), duration of assay (Figure 12B), DTT concentration

10   (Figure 13A), p-NPP concentration (Figure 13B).

One ml assay: 100 µl of GST-cdc25A protein (diluted to an activity of ∂ OD 410 nm = 0.3/10 min) were mixed with 100 µl mM DTT (in Tris buffer A) and 700 µl of Tris buffer A. The assay was initiated by addition of 100 µl

15   500 mM p-NPP (in Tris buffer A). After 10 min incubation at 37°C, the assay was terminated by addition of 40 µl 5 N NaOH and transfer of the tubes to 4°C. Absorbance at 410 nm was then measured and blank values (no GST-cdc25A protein but 10 min incubation) were subtracted.

20   This assay was then scaled down to 200 µl and conducted semi-automatically in 96-wells microtitration plates, as described in detail below. Each well was filled with 20 µl GST-cdc25A phosphatase, 140 µl Tris buffer A, 20 µl 100 mM DTT (in Tris buffer A); after 15

25   min equilibration at 37°C, reaction was initiated by addition of 20 µl 500 mM.p-NPP (in Tris buffer A). After 60 min incubation absorbance at 405 nm was monitored in a microplate reader; blank values (no GST-cdc25A added) were subtracted.

30   Microtitration plate assay: 20 µl of GST-cdc25A protein (diluted to an activity of ∂ OD 405 nm = 0.2-0.3/60 min) were mixed with 20 µl 100 mM DTT (in Tris buffer A) and 140 µl of Tris buffer A, in 96-wells microtitration plates (Corning). The plates were

preincubated at 37°C for 15 min in a Denley Wellwarm 1
microplate incubator. The assays were initiated by
addition of 20 µl of 500 mM p-NPP (in Tris buffer A).
After 60 min incubation at 37°C absorbance at 405 nm was
5   measured in a bioRad microplate reader. Blank values (no
CST-cdc25A protein added) were automatically subtracted.

Example 17   Tyrosine Dephosphorylation and Activation of
the p34^{cdc2}/cyclin B^{cdc13} Kinase by the Fusion
Protein GST-cdc25A

10   The ability of the GST-cdc25A fusion protein to
dephosphorylate and activate the p34^{cdc2}/cyclin B^{cdc13} kinase
was demonstrated. p34^{cdc2}/cyclin B^{cdc13} complex from G2-
arrested starfish oocytes was immobilized on p9^{CKShs1}
agarose: it is constituted of tyrosine-phosphorylated
15   p34^{cdc2} and cyclin B^{cdc13} (Arion, L. et al., Eur. J.
Biochem.: (1992); Pondaven, P. et al., Genes and
Development 4:9-17 (1990)).

Treatment with purified GST-cdc25A protein induced
almost complete tyrosine dephosphorylation of p34^{cdc2} by
20   the p34^{cdc2} mobility shift, the loss of cross-reactivity
with anti-phosphotyrosine antibodies and the appearance of
cross-reactivity with an antibody directed against a
p34^{cdc2} peptide comprising the tyrosine-15 residue (data
not shown). In addition, this tyrosine dephosphorylation
25   lead to histone H1 kinase activation to a level close to
that found in M phase oocytes (Figure 11). By these
criteria, the GST-cdc25A fusion protein appears to display
all the physiological enzymatic activity of cellular
p80^{cdc25}.

30   Assay of p34^{cdc2}/Cyclin B^{cdc13} Kinase Activity

Oocyte extracts were prepared by homogenization of 1
g of G2 or M phase gonads per 2 ml homogenization buffer.
After centrifugation for 10 min at 14,000 g at 4°C, the

supernatants were loaded on p9^{CKShs1}-sepharose beads
prepared as described in Azzi, L. et al. (Eur. J.
Biochem.: in press (1992)) (400 µl supernatant/10 µl
p9^{CKShs1}-beads). The tubes were kept under constant
5   rotation at 4°C for 30 min. After a brief centrifugation
at 10,000 g and removal of the supernatant, the beads were
washed three times with bead buffer and eventually exposed
to purified GST-cdc25A phosphatase prior to H1 kinase
assay or to immunoblotting analysis.

10   Histone H1 kinase assays were performed by incubation
of 10 µl of packed p9^{CKShs1}-beads (loaded with G2 or M phase
extracts) for 10 min at 30°C with 15 µM [γ-32P] ATP (3,000
Ci/mmol; 1 mCi/ml) in the presence of 1 mg histone II1/ml
in a final volume of 40 µl. Assays were terminated by
15   transferring the tube onto ice. After a brief
centrifugation at 10,000 g, 30 µl aliquots of supernatant
were spotted onto 2.5 x 3 cm pieces of Whatman P81
phosphocellulose paper, and after 20 sec, the filters were
washed five times (for at least 5 min each time) in a
20   solution of 10 ml phosphoric acid/liter of water. The wet
filters were transferred into 6 ml plastic scintillation
vials, 5 ml ACS (Amersham) scintillation fluid was added
and the radioactivity of the samples measured in a Packard
counter.

25   Electrophoresis and Western Blotting
Proteins bound to p9^{CKShs1}-sepharose beads were
recovered with 50 µl 2X Laemmli sample buffer. Samples
were run in 10% SDS/polyacrylamide gels. Proteins were
stained with Coomassie Blue or transferred to 0.1 µm
30   nitrocellulose sheets (Schleicher & Schull) in a
Milliblot/SDE system (Millipore) for 30 min at 2.5 mA/cm^2
in transfer buffer. The filters were subsequently blocked
with TBS containing 3% bovine serum albumin for 1 hr at
room temperature. The filters were then incubated

overnight at 4°C with g1 anti-p34cdc2 antibodies (1:1000 dilution), anti-p34[cdc2] peptide antibodies (1:500 dilution) or anti-phosphotyrosine antibodies (1 µg/ml). After four washes of 15 min each with TBS containing 0.2% NP40, the
5   filters were treated with 1 µCi ^{125}I-protein A (30 mCi/mg) in 3% bovine serum albumin in TBS for 2 hr at room temperature. After four 15 min washes with 0.2% NP40 in TBS, the filters were exposed overnight to hyperfilm MP (Amersham).

10 Example 18   Detection of Inhibitors of Purified
                GST-cdc25A Phosphatase
        In a series of experiments various antimitotic compounds currently used in cancer therapy were tested as potential inhibitors of the phosphatase (the Table). Most
15 of them are reported to act as DNA damaging agents, as DNA intercalators, as topoisomerase 2 inhibitors or as compounds interfering with spindle microtubles. None of them displayed GST-cdc25A phosphatase inhibitory activity. As a positive control vanadate, a reported inhibitor of
20 tyrosine phosphatases was also tested (Gordon, J.A., Methods in Enzymology pp. 447-482 (1991)). This compound totally inhibits the GST-cdc25A phosphatase at concentrations above 500 µM (Figure 14; I_{50} = 20 µM).

TABLE

ANTIMITOTIC COMPOUNDS TESTED AS POTENTIAL
INHIBITORS OF P80^{cdc25A}

	Compounds	Range of Concentration Tested
5		
	- Actinomycin D	0.1-100 µg/ml
	- BCNU	0.1-100 µg/ml
	- Carboplatin	0.1-100 µg/ml
10	- Chlormethine	0.1-100 µg/ml
	- Cis-platinum	0.1-100 µg/ml
	- Cyclophosphamide	0.1-100 µg/ml
	- Dacarbazine	0.1-100 µg/ml
	- Doxorubicin	0.1-100 µg/ml
15	- Etoposide	0.1-100 µg/ml
	- Fluoro-uracil	0.1-100 µg/ml
	- Girolline	0.36-360 µg/ml
	- Methotrexate	0.1-100 µg/ml
	- Novobiocin	0.1-100 µg/ml
20	- Vinblastine	0.1-100 µg/ml
	- Vincristine	0.1-100 µg/ml

None of the compounds exhibited more than 5-10% inhibitory activity on the enzyme over the indicated range of

25   concentration.

Equivalents

Those skilled in the art will recognize, or be able
to ascertain using not more than routine experimentation,
nany equivalents to the specific embodiments of the
5   tnvention described herein.   Such equivalents are intended
to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT: Cold Spring Harbor Laboratory

   (ii) TITLE OF INVENTION: Novel Human *cdc25* Genes, Encoded
             Products and Uses Thereof

   (iii) NUMBER OF SEQUENCES: 31

   (iv) CORRESPONDENCE ADDRESS:
           (A) ADDRESSEE: LAHIVE & COCKFIELD
           (B) STREET: 60 State Street
           (C) CITY: Boston
           (D) STATE: Massachusetts
           (E) COUNTRY: U.S.A.
           (F) ZIP: 02109

   (v) COMPUTER READABLE FORM:
           (A) MEDIUM TYPE: Floppy disk
           (B) COMPUTER: IBM PC compatible
           (C) OPERATING SYSTEM: PC-DOS/MS-DOS
           (D) SOFTWARE: ASCII(text)

   (vi) CURRENT APPLICATION DATA:
           (A) APPLICATION NUMBER:
           (B) FILING DATE: 24 April 1995
           (C) CLASSIFICATION:

   (viii) ATTORNEY/AGENT INFORMATION:
           (A) NAME: Vincent, Matthew P.
           (B) REGISTRATION NUMBER: 36,709
           (C) REFERENCE/DOCKET NUMBER: MII-019-DV

   (ix) TELECOMMUNICATION INFORMATION:
           (A) TELEPHONE: 617-227-7400
           (B) TELEFAX: 617-227-5941

(2) INFORMATION FOR SEQ ID NO:1:

   (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 2419 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: double
           (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (ix) FEATURE:
           (A) NAME/KEY: CDS
           (B) LOCATION: 460..2031

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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     GCTACCCGGA GCTGGGCAAG CGGGTTGGGA GAACAGCGAA GACAGCGTGA GCCTGGGCCG        120

     TTGCCTCGAG GCTCTCGCCC GGCTTCTCTT GCCGACCCGC CACGTTTGTT TGGATTTAAT        180

     CTTACAGCTG GTTGCCGGCG CCCGCCCGCC CGCTGGCCTC GCGGTGTGAG AGGGAAGCAC        240

     CCGTGCCTGT GGCTGGTGGC TGGCGCCTGG AGGGTCCGCA CACCCGCCCG GCCGCGCCGC        300

     TTTGCCCGCG GCAGCCGCGT CCCTGAACCG CGGAGTCGTG TTTGTGTTTG ACCCGCGGGC        360

     GCCGGTGGCG CGCGGCCGAG GCCGGTGTCG GCGGGGCGGG GCGGTCGCGG CGGAGGCAGA        420

     GGAAGAGGGA GCGGGAGCTC TGCGAGGCCG GGCGCCGCC ATG GAA CTG GGC CCG          474
                                                 Met Glu Leu Gly Pro
                                                  1               5
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     AGC CCC GCA CCG CGC CGC CTG CTC TTC GCC TGC AGC CCC CCT CCC GCG        522
     Ser Pro Ala Pro Arg Arg Leu Leu Phe Ala Cys Ser Pro Pro Pro Ala
                      10            15                  20

     TCG CAG CCC GTC GTG AAG GCG CTA TTT GGC GCT TCA GCC GCC GGG GGA        570
     Ser Gln Pro Val Val Lys Ala Leu Phe Gly Ala Ser Ala Ala Gly Gly
                  25            30            35

     CTG TCG CCT GTC ACC AAC CTG ACC GTC ACT ATG GAC CAG CTG CAG GGT        618
     Leu Ser Pro Val Thr Asn Leu Thr Val Thr Met Asp Gln Leu Gln Gly
              40            45            50

     CTG GGC AGT GAT TAT GAG CAA CCA CTG GAG GTG AAG AAC AAC AGT AAT        666
     Leu Gly Ser Asp Tyr Glu Gln Pro Leu Glu Val Lys Asn Asn Ser Asn
          55            60            65

     CTG CAG AGA ATG GGC TCC TCC GAG TCA ACA GAT TCA GGT TTC TGT CTA        714
     Leu Gln Arg Met Gly Ser Ser Glu Ser Thr Asp Ser Gly Phe Cys Leu
      70            75            80            85

     GAT TCT CCT GGG CCA TTG GAC AGT AAA GAA AAC CTT GAA AAT CCT ATG        762
     Asp Ser Pro Gly Pro Leu Asp Ser Lys Glu Asn Leu Glu Asn Pro Met
                  90            95            100

     AGA AGA ATA CAT TCC CTA CCT CAA AAG CTG TTG GGA TGT AGT CCA GCT        810
     Arg Arg Ile His Ser Leu Pro Gln Lys Leu Leu Gly Cys Ser Pro Ala
                  105           110           115

     CTG AAG AGG AGC CAT TCT GAT TCT CTT GAC CAT GAC ATC TTT CAG CTC        858
     Leu Lys Arg Ser His Ser Asp Ser Leu Asp His Asp Ile Phe Gln Leu
                  120           125           130

     ATC GAC CCA GAT GAG AAC AAG GAA AAT GAA GCC TTT GAG TTT AAG AAG        906
     Ile Asp Pro Asp Glu Asn Lys Glu Asn Glu Ala Phe Glu Phe Lys Lys
                  135           140           145

     CCA GTA AGA CCT GTA TCT CGT GGC TGC CTG CAC TCT CAT GGA CTC CAG        954
     Pro Val Arg Pro Val Ser Arg Gly Cys Leu His Ser His Gly Leu Gln
      150           155           160           165

     GAG GGT AAA GAT CTC TTC ACA CAG AGG CAG AAC TCT GCC CAG CTC GGA       1002
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      Glu Gly Lys Asp Leu Phe Thr Gln Arg Gln Asn Ser Ala Gln Leu Gly
                   170               175              180

      ATG CTT TCC TCA AAT GAA AGA GAT AGC AGT GAA CCA GGG AAT TTC ATT      1050
   5  Met Leu Ser Ser Asn Glu Arg Asp Ser Ser Glu Pro Gly Asn Phe Ile
                   185               190              195

      CCT CTT TTT ACA CCC CAG TCA CCT GTG ACA GCC ACT TTG TCT GAT GAG      1098
      Pro Leu Phe Thr Pro Gln Ser Pro Val Thr Ala Thr Leu Ser Asp Glu
  10               200               205              210

      GAT GAT GGC TTC GTG GAC CTT CTC GAT GGA GAG AAT CTG AAG AAT GAG      1146
      Asp Asp Gly Phe Val Asp Leu Leu Asp Gly Glu Asn Leu Lys Asn Glu
                   215               220              225
  15
      GAG GAG ACC CCC TCG TGC ATG GCA AGC CTC TGG ACA GCT CCT CTC GTC      1194
      Glu Glu Thr Pro Ser Cys Met Ala Ser Leu Trp Thr Ala Pro Leu Val
      230               235              240               245

  20  ATG AGA ACT ACA AAC CTT GAC AAC CGA TGC AAG CTG TTT GAC TCC CCT      1242
      Met Arg Thr Thr Asn Leu Asp Asn Arg Cys Lys Leu Phe Asp Ser Pro
                   250               255              260

      TCC CTG TGT AGC TCC AGC ACT CGG TCA GTG TTG AAG AGA CCA GAA CGT      1290
  25  Ser Leu Cys Ser Ser Ser Thr Arg Ser Val Leu Lys Arg Pro Glu Arg
                   265               270              275

      TCT CAA GAG GAG TCT CCA CCT GGA AGT ACA AAG AGG AGG AAG AGC ATG      1338
      Ser Gln Glu Glu Ser Pro Pro Gly Ser Thr Lys Arg Arg Lys Ser Met
  30               280               285              290

      TCT GGG GCC AGC CCC AAA GAG TCA ACT AAT CCA GAG AAG GCC CAT GAG      1386
      Ser Gly Ala Ser Pro Lys Glu Ser Thr Asn Pro Glu Lys Ala His Glu
                   295               · 300              305
  35
      ACT CTT CAT CAG TCT TTA TCC CTG GCA TCT TCC CCC AAA GGA ACC ATT      1434
      Thr Leu His Gln Ser Leu Ser Leu Ala Ser Ser Pro Lys Gly Thr Ile
      310               315              320               325

  40  GAG AAC ATT TTG GAC AAT GAC CCA AGG GAC CTT ATA GGA GAC TTC TCC      1482
      Glu Asn Ile Leu Asp Asn Asp Pro Arg Asp Leu Ile Gly Asp Phe Ser
                   330               335              340

      AAG GGT TAT CTC TTT CAT ACA GTT GCT GGG AAA CAT CAG GAT TTA AAA      1530
  45  Lys Gly Tyr Leu Phe His Thr Val Ala Gly Lys His Gln Asp Leu Lys
                   345               350              355

      TAC ATC TCT CCA GAA ATT ATG GCA TCT GTT TTG AAT GGC AAG TTT GCC      1578
      Tyr Ile Ser Pro Glu Ile Met Ala Ser Val Leu Asn Gly Lys Phe Ala
  50               360               365              370

      AAC CTC ATT AAA GAG TTT GTT ATC ATC GAC TGT CGA TAC CCA TAT GAA      1626
      Asn Leu Ile Lys Glu Phe Val Ile Ile Asp Cys Arg Tyr Pro Tyr Glu
                   375               380              385
  55
      TAC GAG GGA GGC CAC ATC AAG GGT GCA GTG AAC TTG CAC ATG GAA GAA      1674
      Tyr Glu Gly Gly His Ile Lys Gly Ala Val Asn Leu His Met Glu Glu
      390               395              400               405
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GAG GTT GAA GAC TTC TTA TTG AAG AAG CCC ATT GTA CCT ACT GAT GGC          1722
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            410                     415                 420

AAG CGT GTC ATT GTT GTG TTT CAC TGC GAG TTT TCT TCT GAG AGA GGT          1770
Lys Arg Val Ile Val Val Phe His Cys Glu Phe Ser Ser Glu Arg Gly
            425                     430                 435

CCC CGC ATG TGC CGG TAT GTG AGA GAG AGA GAT CGC CTG GGT AAT GAA          1818
Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp Arg Leu Gly Asn Glu
            440                     445                 450

TAC CCC AAA CTC CAC TAC CCT GAG CTG TAT GTC CTG AAG GGG GGA TAC          1866
Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val Leu Lys Gly Gly Tyr
        455                     460                 465

AAG GAG TTC TTT ATG AAA TGC CAG TCT TAC TGT GAG CCC CCT AGC TAC          1914
Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys Glu Pro Pro Ser Tyr
    470                     475                 480                 485

CGG CCC ATG CAC CAC GAG GAC TTT AAA GAA GAC CTG AAG AAG TTC CGC          1962
Arg Pro Met His His Glu Asp Phe Lys Glu Asp Leu Lys Lys Phe Arg
                490                     495                 500

ACC AAG AGC CGG ACC TGG GCA GGG GAG AAG AGC AAG AGG GAG ATG TAC          2010
Thr Lys Ser Arg Thr Trp Ala Gly Glu Lys Ser Lys Arg Glu Met Tyr
                505                     510                 515

AGT CGT CTG AAG AAG CTC TGAGGGCGGC AGGACCAGCC AGCAGCAGCC                 2058
Ser Arg Leu Lys Lys Leu
                520

CAAGCTTCCC TCCATCCCCC TTTACCCTCT TTCCTGCAGA GAAACTTAAG CAAAGGGGAC       2118

AGCTGTGTGA CATTTGGAGA GGGGGCCTGG GACTTCCATG CCTTAAACCT ACCTCCCACA       2178

CTCCCAAGGT TGGAGCCCAG GGCATCTTGC TGGCTACGCC TCTTCTGTCC CTGTTAGACG       2238

TCCTCCGTCC ATATCAGAAC TGTGCCACAA TGCAGTTCTG AGCACCGTGT CAAGCTGCTC       2298

TGAGCCACAG TGGGATGAAC CAGCCGGGGC CTTATCGGGC TCCAGCATCT CATGAGGGGA       2358

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G                                                                       2419
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(2) INFORMATION FOR SEQ ID NO:2:

     (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 523 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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   5  Ser Pro Pro Pro Ala Ser Gln Pro Val Val Lys Ala Leu Phe Gly Ala
                    20                  25                  30

      Ser Ala Ala Gly Gly Leu Ser Pro Val Thr Asn Leu Thr Val Thr Met
                35                  40                  45
  10
      Asp Gln Leu Gln Gly Leu Gly Ser Asp Tyr Glu Gln Pro Leu Glu Val
              50                  55                  60

      Lys Asn Asn Ser Asn Leu Gln Arg Met Gly Ser Ser Glu Ser Thr Asp
  15   65                  70                  75                  80

      Ser Gly Phe Cys Leu Asp Ser Pro Gly Pro Leu Asp Ser Lys Glu Asn
                    85                  90                  95

  20  Leu Glu Asn Pro Met Arg Arg Ile His Ser Leu Pro Gln Lys Leu Leu
                    100                 105                 110

      Gly Cys Ser Pro Ala Leu Lys Arg Ser His Ser Asp Ser Leu Asp His
                115                 120                 125
  25
      Asp Ile Phe Gln Leu Ile Asp Pro Asp Glu Asn Lys Glu Asn Glu Ala
                130                 135                 140

      Phe Glu Phe Lys Lys Pro Val Arg Pro Val Ser Arg Gly Cys Leu His
  30  145                 150                 155                 160

      Ser His Gly Leu Gln Glu Gly Lys Asp Leu Phe Thr Gln Arg Gln Asn
                    165                 170                 175

  35  Ser Ala Gln Leu Gly Met Leu Ser Ser Asn Glu Arg Asp Ser Ser Glu
                180                 185                 190

      Pro Gly Asn Phe Ile Pro Leu Phe Thr Pro Gln Ser Pro Val Thr Ala
                195                 200                 205
  40
      Thr Leu Ser Asp Glu Asp Asp Gly Phe Val Asp Leu Leu Asp Gly Glu
                210                 215                 220

      Asn Leu Lys Asn Glu Glu Glu Thr Pro Ser Cys Met Ala Ser Leu Trp
  45  225                 230                 235                 240

      Thr Ala Pro Leu Val Met Arg Thr Thr Asn Leu Asp Asn Arg Cys Lys
                    245                 250                 255

  50  Leu Phe Asp Ser Pro Ser Leu Cys Ser Ser Ser Thr Arg Ser Val Leu
                    260                 265                 270

      Lys Arg Pro Glu Arg Ser Gln Glu Glu Ser Pro Pro Gly Ser Thr Lys
                275                 280                 285
  55
      Arg Arg Lys Ser Met Ser Gly Ala Ser Pro Lys Glu Ser Thr Asn Pro
                290                 295                 300
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      Glu Lys Ala His Glu Thr Leu His Gln Ser Leu Ser Leu Ala Ser Ser
      305                 310             315             320

  5   Pro Lys Gly Thr Ile Glu Asn Ile Leu Asp Asn Asp Pro Arg Asp Leu
                      325             330             335

      Ile Gly Asp Phe Ser Lys Gly Tyr Leu Phe His Thr Val Ala Gly Lys
                      340             345             350

 10   His Gln Asp Leu Lys Tyr Ile Ser Pro Glu Ile Met Ala Ser Val Leu
                      355             360             365

      Asn Gly Lys Phe Ala Asn Leu Ile Lys Glu Phe Val Ile Ile Asp Cys
                  370             375             380
 15
      Arg Tyr Pro Tyr Glu Tyr Glu Gly Gly His Ile Lys Gly Ala Val Asn
      385                 390             395             400

      Leu His Met Glu Glu Glu Val Glu Asp Phe Leu Leu Lys Lys Pro Ile
 20                       405             410             415

      Val Pro Thr Asp Gly Lys Arg Val Ile Val Val Phe His Cys Glu Phe
                      420             425             430

 25   Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp
                  435             440             445

      Arg Leu Gly Asn Glu Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val
              450             455             460
 30
      Leu Lys Gly Gly Tyr Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys
      465                 470             475             480

 35   Glu Pro Pro Ser Tyr Arg Pro Met His His Glu Asp Phe Lys Glu Asp
                      485             490             495

      Leu Lys Lys Phe Arg Thr Lys Ser Arg Thr Trp Ala Gly Glu Lys Ser
                  500             505             510

 40   Lys Arg Glu Met Tyr Ser Arg Leu Lys Lys Leu
              515             520


      (2)  INFORMATION FOR SEQ ID NO:3:
 45
              (i)  SEQUENCE CHARACTERISTICS:
                  (A)  LENGTH: 2940 base pairs
                  (B)  TYPE: nucleic acid
                  (C)  STRANDEDNESS: double
 50               (D)  TOPOLOGY: linear

             (ii)  MOLECULE TYPE: DNA (genomic)

             (ix)  FEATURE:
 55               (A)  NAME/KEY: CDS
                  (B)  LOCATION: 73..1773

             (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:3:
```

```
CTGCCCTGCG CCCGGCCCTC CAGCCAGCCT GCCAGCTGTG CCGGCGTTTG TTGGTCTGCC          60


GGCCCCGCCG CG ATG GAG GTG CCC CAG CCG GAG CCC GCG CCA GGC TCG          108
              Met Glu Val Pro Gln Pro Glu Pro Ala Pro Gly Ser
               1                 5                    10


GCT CTC AGT CCA GCA GGC GTG TGC GGT GGC GCC CAG CGT CCG GGC CAC          156
Ala Leu Ser Pro Ala Gly Val Cys Gly Gly Ala Gln Arg Pro Gly His
         15                  20                  25


CTC CCG GGC CTC CTG CTG GGA TCT CAT GGC CTC CTG GGG TCC CCG GTG          204
Leu Pro Gly Leu Leu Leu Gly Ser His Gly Leu Leu Gly Ser Pro Val
     30                  35                  40


CGG GCG GCC GCT TCC TCG CCG GTC ACC ACC CTC ACC CAG ACC ATG CAC          252
Arg Ala Ala Ala Ser Ser Pro Val Thr Thr Leu Thr Gln Thr Met His
 45              50                  55                  60


GAC CTC GCC GGG CTC GGC AGC CGC AGC CGC CTG ACG CAC CTA TCC CTG          300
Asp Leu Ala Gly Leu Gly Ser Arg Ser Arg Leu Thr His Leu Ser Leu
                 65                  70                  75


TCT CGA CGG GCA TCC GAA TCC TCC CTG TCG TCT GAA TCC TCC GAA TCT          348
Ser Arg Arg Ala Ser Glu Ser Ser Leu Ser Ser Glu Ser Ser Glu Ser
             80                  85                  90


TCT GAT GCA GGT CTC TGC ATG GAT TCC CCC AGC CCT ATG GAC CCC CAC          396
Ser Asp Ala Gly Leu Cys Met Asp Ser Pro Ser Pro Met Asp Pro His
         95                  100                 105


ATG GCG GAG CAG ACG TTT GAA CAG GCC ATC CAG GCA GCC AGC CGG ATC          444
Met Ala Glu Gln Thr Phe Glu Gln Ala Ile Gln Ala Ala Ser Arg Ile
     110             115                 120


ATT CGA AAC GAG CAG TTT GCC ATC AGA CGC TTC CAG TCT ATG CCG GTG          492
Ile Arg Asn Glu Gln Phe Ala Ile Arg Arg Phe Gln Ser Met Pro Val
125             130                 135                 140


AGG CTG CTG GGC CAC AGC CCC GTG CTT CGG AAC ATC ACC AAC TCC CAG          540
Arg Leu Leu Gly His Ser Pro Val Leu Arg Asn Ile Thr Asn Ser Gln
             145                 150                 155


GCG CCC GAC GGC CGG AGG AAG AGC GAG GCG GGC AGT GGA GCT GCC AGC          588
Ala Pro Asp Gly Arg Arg Lys Ser Glu Ala Gly Ser Gly Ala Ala Ser
             160                 165                 170


AGC TCT GGG GAA GAC AAG GAG AAT GAT GGA TTT GTC TTC AAG ATG CCA          636
Ser Ser Gly Glu Asp Lys Glu Asn Asp Gly Phe Val Phe Lys Met Pro
         175                 180                 185


TGG AAG CCC ACA CAT CCC AGC TCC ACC CAT GCT CTG GCA GAG TGG GCC          684
Trp Lys Pro Thr His Pro Ser Ser Thr His Ala Leu Ala Glu Trp Ala
         190             195                 200


AGC CGC AGG GAA GCC TTT GCC CAG AGA CCC AGC TCG GCC CCC GAC CTG          732
Ser Arg Arg Glu Ala Phe Ala Gln Arg Pro Ser Ser Ala Pro Asp Leu
205             210                 215                 220
```

```
ATG TGT CTC AGT CCT GAC CGG AAG ATG GAA GTG GAG GAG CTC AGC CCC          780
Met Cys Leu Ser Pro Asp Arg Lys Met Glu Val Glu Glu Leu Ser Pro
            225             230                 235

CTG GCC CTA GGT CGC TTC TCT CTG ACC CCT GCA GAG GGG GAT ACT GAG          828
Leu Ala Leu Gly Arg Phe Ser Leu Thr Pro Ala Glu Gly Asp Thr Glu
            240             245                 250

GAA GAT GAT GGA TTT GTG GAC ATC CTA GAG AGT GAC TTA AAG GAT GAT          876
Glu Asp Asp Gly Phe Val Asp Ile Leu Glu Ser Asp Leu Lys Asp Asp
            255             260                 265

GAT GCA GTT CCC CCA GGC ATG GAG AGT CTC ATT AGT GCC CCA CTG GTC          924
Asp Ala Val Pro Pro Gly Met Glu Ser Leu Ile Ser Ala Pro Leu Val
            270             275                 280

AAG ACC TTG GAA AAG GAA GAG GAA AAG GAC CTC GTC ATG TAC AGC AAG          972
Lys Thr Leu Glu Lys Glu Glu Glu Lys Asp Leu Val Met Tyr Ser Lys
            285             290                 295             300

TGC CAG CGG CTC TTC CGC TCT CCG TCC ATG CCC TGC AGC GTG ATC CGG         1020
Cys Gln Arg Leu Phe Arg Ser Pro Ser Met Pro Cys Ser Val Ile Arg
                305                 310                 315

CCC ATC CTC AAG AGG CTG GAG CGG CCC CAG GAC AGG GAC ACG CCC GTG         1068
Pro Ile Leu Lys Arg Leu Glu Arg Pro Gln Asp Arg Asp Thr Pro Val
                320                 325                 330

CAG AAT AAG CGG AGG CGG AGC GTG ACC CCT CCT GAG GAG CAG CAG GAG         1116
Gln Asn Lys Arg Arg Arg Ser Val Thr Pro Pro Glu Glu Gln Gln Glu
            335             340                 345

GCT GAG GAA CCT AAA GCC CGC GTC CTC CGC TCA AAA TCA CTG TGT CAC         1164
Ala Glu Glu Pro Lys Ala Arg Val Leu Arg Ser Lys Ser Leu Cys His
            350             355                 360

GAT GAG ATC GAG AAC CTC CTG GAC AGT GAC CAC CGA GAG CTG ATT GGA         1212
Asp Glu Ile Glu Asn Leu Leu Asp Ser Asp His Arg Glu Leu Ile Gly
365             370                 375                 380

GAT TAC TCT AAG GCC TTC CTC CTA CAG ACA GTA GAC GGA AAG CAC CAA         1260
Asp Tyr Ser Lys Ala Phe Leu Leu Gln Thr Val Asp Gly Lys His Gln
                385             390                 395

GAC CTC AAG TAC ATC TCA CCA GAA ACG ATG GTG GCC CTA TTG ACG GGC         1308
Asp Leu Lys Tyr Ile Ser Pro Glu Thr Met Val Ala Leu Leu Thr Gly
                400             405                 410

AAG TTC AGC AAC ATC GTG GAT AAG TTT GTG ATT GTA GAC TGC AGA TAC         1356
Lys Phe Ser Asn Ile Val Asp Lys Phe Val Ile Val Asp Cys Arg Tyr
            415             420                 425

CCC TAT GAA TAT GAA GGC GGG CAC ATC AAG ACT GCG GTG AAC TTG CCC         1404
Pro Tyr Glu Tyr Glu Gly Gly His Ile Lys Thr Ala Val Asn Leu Pro
            430             435                 440

CTG GAA CGC GAC GCC GAG AGC TTC CTA CTG AAG AGC CCC ATC GCG CCC         1452
```

```
        Leu Glu Arg Asp Ala Glu Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro
        445             450                 455                 460

        TGT AGC CTG GAC AAG AGA GTC ATC CTC ATT TTC CAC TGT GAA TTC TCA        1500
  5     Cys Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser
                            465                 470                 475

        TCT GAG CGT GGG CCC CGC ATG TGC CGT TTC ATC AGG GAA CGA GAC CGT        1548
        Ser Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg
 10                     480                 485                 490

        GCT GTC AAC GAC TAC CCC AGC CTC TAC TAC CCT GAG ATG TAT ATC CTG        1596
        Ala Val Asn Asp Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile Leu
 15                     495                 500                 505

        AAA GGC GGC TAC AAG GAG TTC TTC CCT CAG CAC CCG AAC TTC TGT GAA        1644
        Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys Glu
                510                 515                 520

 20     .CCC CAG GAC TAC CGG @CC ATG AAC CAC GAG GCC TTC AAG GAT GAG CTA       1692
        Pro Gln Asp Tyr Arg Pro Met Asn His Glu Ala Phe Lys Asp Glu Leu
        525                 530                 535                 540

        AAG ACC TTC CGC CTC AAG ACT CGC AGC TGG GCT GGG GAG CGG AGC CGG        1740
 25     Lys Thr Phe Arg Leu Lys Thr Arg Ser Trp Ala Gly Glu Arg Ser Arg
                            545                 550                 555

        CGG GAG CTC TGT AGC CGG CTG CAG GAC CAG TGAGGGGCCT GCGCCAGTCC          1790
        Arg Glu Leu Cys Ser Arg Leu Gln Asp Gln
 30                     560                 565

        TGCTACCTCC CTTGCCTTTC GAGGCCTGAA GCCAGCTGCC CTATGGGCCT GCCGGGCTGA      1850

        GGGCCTGCTG GAGGCCTCAG GTGCTGTCCA TGGGAAAGAT GGTGTGGTGT CCTGCCTGTC      1910

 35     TGCCCCAGCC CAGATTCCCC TGTGTCATCC CATCATTTTC CATATCCTGG TGCCCCCCAC      1970

        CCCTGGAAGA GCCCAGTCTG TTGAGTTAGT TAAGTTGGGT TAATACCAGC TTAAAGGCAG      2030

 40     TATTTTGTGT CCTCCAGGAG CTTCTTGTTT CCTTGTTAGG GTTAACCCTT CATCTTCCTG      2090

        TGTCCTGAAA CGCTCCTTTG TGTGTGTGTC AGCTGAGGCT GGGGAGAGCC GTGGTCCCTG      2150

        AGGATGGGTC AGAGCTAAAC TCCTTCCTGG CCTGAGAGTC AGCTCTCTGC CCTGTGTACT      2210

 45     TCCCGGGCCA GGGCTGCCCC TAATCTCTGT AGGAACCGTG GTATGTCTGC CATGTTGCCC      2270

        CTTTCTCTTT TCCCCTTTCC TGTCCCACCA TACGAGCACC TCCAGCCTGA ACAGAAGCTC      2330

 50     TTACTCTTTC CTATTTCAGT GTTACCTGTG TGCTTGGTCT GTTTGACTTT ACGCCCATCT      2390

        CAGGACACTT CCGTAGACTG TTTAGGTTCC CCTGTCAAAT ATCAGTTACC CACTCGGTCC      2450

        CAGTTTTGTT GCCCCAGAAA GGGATGTTAT TATCCTTGGG GGCTCCCAGG GCAAGGGTTA      2510

 55     AGGCCTGAAT CATGAGCCTG CTGGAAGCCC AGCCCCTACT GCTGTGAACC CTGGGGCCTG      2570

        ACTGCTCAGA ACTTGCTGCT GTCTTGTTGC GGATGGATGG AAGGTTGGAT GGATGGGTGG      2630
```

```
ATGGCCGTGG ATGGCCGTGG ATGCGCAGTG CCTTGCATAC CCAAACCAGG TGGGAGCGTT    2690

TTGTTGAGCA TGACACCTGC AGCAGGAATA TATGTGTGCC TATTTGTGTG GACAAAAATA    2750

TTTACACTTA GGGTTTGGAG CTATTCAAGA GGAAATGTCA CAGAAGCAGC TAAACCAAGG    2810

ACTGAGCACC CTCTGGATTC TGAATCTCAA GATGGGGGCA GGGCTGTGCT TGAAGGCCCT    2870

GCTGAGTCAT CTGTTAGGGC CTTGGTTCAA TAAAGCACTG AGCAAGTTGA GAAAAAAAAA    2930

AAAAAAAAAA                                                          2940
```

(2) INFORMATION FOR SEQ ID NO:4:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 566 amino acids
         (B) TYPE: amino acid
         (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Glu Val Pro Gln Pro Glu Pro Ala Pro Gly Ser Ala Leu Ser Pro
 1               5                  10                  15

Ala Gly Val Cys Gly Gly Ala Gln Arg Pro Gly His Leu Pro Gly Leu
            20                  25                  30

Leu Leu Gly Ser His Gly Leu Leu Gly Ser Pro Val Arg Ala Ala Ala
            35                  40                  45

Ser Ser Pro Val Thr Thr Leu Thr Gln Thr Met His Asp Leu Ala Gly
        50                  55                  60

Leu Gly Ser Arg Ser Arg Leu Thr His Leu Ser Leu Ser Arg Arg Ala
 65                  70                  75                  80

Ser Glu Ser Ser Leu Ser Ser Glu Ser Ser Glu Ser Ser Asp Ala Gly
                85                  90                  95

Leu Cys Met Asp Ser Pro Ser Pro Met Asp Pro His Met Ala Glu Gln
            100                 105                 110

Thr Phe Glu Gln Ala Ile Gln Ala Ala Ser Arg Ile Ile Arg Asn Glu
        115                 120                 125

Gln Phe Ala Ile Arg Arg Phe Gln Ser Met Pro Val Arg Leu Leu Gly
        130                 135                 140

His Ser Pro Val Leu Arg Asn Ile Thr Asn Ser Gln Ala Pro Asp Gly
145                 150                 155                 160

Arg Arg Lys Ser Glu Ala Gly Ser Gly Ala Ala Ser Ser Ser Gly Glu
                165                 170                 175
```

```
Asp Lys Glu Asn Asp Gly Phe Val Phe Lys Met Pro Trp Lys Pro Thr
            180                 185                 190
```

```
His Pro Ser Ser Thr His Ala Leu Ala Glu Trp Ala Ser Arg Arg Glu
            195                 200                 205
```

```
Ala Phe Ala Gln Arg Pro Ser Ser Ala Pro Asp Leu Met Cys Leu Ser
    210                 215                 220
```

```
Pro Asp Arg Lys Met Glu Val Glu Glu Leu Ser Pro Leu Ala Leu Gly
225                 230                 235                 240
```

```
Arg Phe Ser Leu Thr Pro Ala Glu Gly Asp Thr Glu Glu Asp Asp Gly
                245                 250                 255
```

```
Phe Val Asp Ile Leu Glu Ser Asp Leu Lys Asp Asp Asp Ala Val Pro
            260                 265                 270
```

```
Pro Gly Met Glu Ser Leu Ile Ser Ala Pro Leu Val Lys Thr Leu Glu
            275                 280                 285
```

```
Lys Glu Glu Glu Lys Asp Leu Val Met Tyr Ser Lys Cys Gln Arg Leu
        290                 295                 300
```

```
Phe Arg Ser Pro Ser Met Pro Cys Ser Val Ile Arg Pro Ile Leu Lys
305                 310                 315                 320
```

```
Arg Leu Glu Arg Pro Gln Asp Arg Asp Thr Pro Val Gln Asn Lys Arg
                325                 330                 335
```

```
Arg Arg Ser Val Thr Pro Pro Glu Glu Gln Gln Glu Ala Glu Glu Pro
            340                 345                 350
```

```
Lys Ala Arg Val Leu Arg Ser Lys Ser Leu Cys His Asp Glu Ile Glu
            355                 360                 365
```

```
Asn Leu Leu Asp Ser Asp His Arg Glu Leu Ile Gly Asp Tyr Ser Lys
    370                 375                 380
```

```
Ala Phe Leu Leu Gln Thr Val Asp Gly Lys His Gln Asp Leu Lys Tyr
385                 390                 395                 400
```

```
Ile Ser Pro Glu Thr Met Val Ala Leu Leu Thr Gly Lys Phe Ser Asn
                405                 410                 415
```

```
Ile Val Asp Lys Phe Val Ile Val Asp Cys Arg Tyr Pro Tyr Glu Tyr
            420                 425                 430
```

```
Glu Gly Gly His Ile Lys Thr Ala Val Asn Leu Pro Leu Glu Arg Asp
        435                 440                 445
```

```
Ala Glu Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro Cys Ser Leu Asp
    450                 455                 460
```

```
Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser Ser Glu Arg Gly
465                 470                 475                 480
```

```
Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg Ala Val Asn Asp
```

```
                485                  490                  495
    Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile Leu Lys Gly Gly Tyr
                500                  505                  510
 5
    Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys Glu Pro Gln Asp Tyr
                515                  520                  525

    Arg Pro Met Asn His Glu Ala Phe Lys Asp Glu Leu Lys Thr Phe Arg
10              530                  535                  540

    Leu Lys Thr Arg Ser Trp Ala Gly Glu Arg Ser Arg Arg Glu Leu Cys
    545                  550                  555                  560

15  Ser Arg Leu Gln Asp Gln
                565
```

    (2) INFORMATION FOR SEQ ID NO:5:
20
         (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 205 amino acids
              (B) TYPE: amino acid
              (D) TOPOLOGY: linear

25   (ii) MOLECULE TYPE: peptide

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
    Leu Asp Asn Asp Pro Arg Asp Leu Ile Gly Asp Phe Ser Lys Gly Tyr
    1                 5                 10                  15

    Leu Phe His Thr Val Ala Gly Lys His Gln Asp Leu Lys Tyr Ile Ser
                20                   25                  30

    Pro Glu Ile Met Ala Ser Val Leu Asn Gly Lys Phe Ala Asn Leu Ile
                35                   40                 45

    Lys Glu Phe Val Ile Ile Asp Cys Arg Tyr Pro Tyr Glu Tyr Glu Gly
                50                   55                  60

    Gly His Ile Lys Gly Ala Val Asn Leu His Met Glu Glu Glu Val Glu
    65                   70                   75                  80

    Asp Phe Leu Leu Lys Lys Pro Ile Val Pro Xaa Xaa Xaa Xaa Xaa Xaa
                85                   90                  95

    Xaa Xaa Thr Asp Gly Lys Arg Val Ile Val Val Phe His Cys Glu Phe
                100                  105                 110

    Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp
                115                  120                 125

    Arg Leu Gly Asn Glu Xaa Xaa Tyr Pro Lys Leu His Tyr Pro Glu Leu
                130                  135                 140

    Tyr Val Leu Lys Gly Gly Tyr Lys Glu Phe Phe Met Lys Cys Gln Ser
    145                  150                  155                 160
```

```
        Tyr Cys Glu Pro Pro Ser Tyr Arg Pro Met His His Glu Asp Phe Lys
                        165                 170                 175

        Glu Asp Leu Lys Lys Phe Arg Thr Lys Ser Arg Thr Trp Ala Gly Glu
                        180                 185                 190

        Lys Ser Lys Arg Glu Met Tyr Ser Arg Leu Lys Lys Leu
                        195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 205 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE; peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
        Leu Asp Ser Asp His Arg Glu Leu Ile Gly Asp Tyr Ser Lys Ala Phe
        1               5                   10                  15

        Leu Leu Gln Thr Val Asp Gly Lys His Gln Asp Leu Lys Tyr Ile Ser
                        20                  25                  30

        Pro Glu Thr Val Met Ala Leu Leu Thr Gly Lys Phe Ser Asn Ile Val
                        35                  40                  45

        Asp Lys Phe Val Ile Val Asp Cys Arg Tyr Pro Tyr Glu Tyr Glu Gly
                50                  55                  60

        Gly His Ile Lys Thr Ala Val Asn Leu Pro Leu Glu Arg Asp Ala Glu
        65                  70                  75                  80

        Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro Cys Xaa Xaa Xaa Xaa Xaa
                        85                  90                  95

        Xaa Xaa Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe
                        100                 105                 110

        Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp
                        115                 120                 125

        Arg Ala Val Asn Asp Xaa Xaa Tyr Pro Ser Leu Tyr Tyr Pro Glu Met
                        130                 135                 140

        Tyr Ile Leu Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn
        145                 150                 155                 160

        Phe Cys Glu Pro Gln Asp Tyr Arg Pro Met Asn His Glu Ala Phe Lys
                        165                 170                 175

        Asp Glu Leu Lys Thr Phe Arg Leu Lys Thr Arg Ser Trp Ala Gly Glu
                        180                 185                 190
```

```
            Arg Ser Arg Arg Glu Leu Cys Ser Arg Leu Gln Asp Gln
                    195             200             205
```

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 208 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: peptide

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Glu Asp Ser Asn Gln Gly His Leu Ile Gly Asp Phe Ser Lys Val Cys
1               5               10              15

Ala Leu Pro Thr Val Ser Gly Lys His Gln Asp Leu Lys Tyr Val Asn
            20              25              30

Pro Glu Thr Val Ala Ala Leu Leu Ser Gly Lys Phe Gln Gly Leu Ile
            35          .       40              45

Glu Lys Phe Tyr Val Ile Asp Cys Arg Tyr Pro Tyr Glu Tyr Leu Gly
        50              55              60  .

Gly His Ile Gln Gly Ala Leu Asn Leu Tyr Ser Gln Glu Glu Leu Phe
65              70              75              80

Asn Phe Phe Leu Lys Lys Pro Ile Val Pro Leu Xaa Xaa Xaa Xaa Xaa
                85              90              95

Xaa Xaa Asp Thr Gln Lys Arg Ile Ile Ile Val Phe His Cys Glu Phe
            100             105             110

Ser Ser Glu Arg Gly Pro Arg Met Cys Arg Cys Leu Arg Glu Glu Asp
            115             120             125

Arg Ser Leu Asn Gln Xaa Xaa Tyr Pro Ala Leu Tyr Tyr Pro Glu Leu
            130             135             140

Tyr Ile Leu Lys Gly Gly Tyr Arg Asp Phe Phe Pro Glu Tyr Met Glu
145             150             155             160

Leu Cys Glu Pro Gln Ser Tyr Cys Pro Met His His Gln Asp His Lys
                165             170             175

Thr Glu Leu Leu Arg Cys Arg Ser Gln Ser Lys Val Gln Glu Gly Glu
            180             185             190

Arg Gln Leu Arg Glu Gln Ile Ala Leu Leu Val Lys Asp Met Ser Pro
            195             200             205
```

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 211 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Glu Asn Arg Asn Glu Pro Glu Leu Ile Gly Asp Phe Ser Lys Ala Tyr
1               5                   10                  15

Ser Leu Pro Leu Met Glu Gly Arg His Arg Asp Leu Lys Ser Ile Ser
            20                  25                  30

Ser Glu Thr Val Ala Arg Leu Leu Lys Gly Glu Phe Ser Asp Lys Val
            35                  40                  45

Ala Ser Tyr Arg Ile Ile Asp Cys Arg Tyr Pro Tyr Glu Phe Glu Gly
        50                  55                  60

Gly His Ile Glu Gly Ala Lys Asn Leu Tyr Thr Thr Glu Gln Ile Leu
65                  70                  75                  80

Asp Glu Phe Leu Thr Val Gln Gln Thr Glu Leu Gln Gln Gln Gln Asn
                85                  90                  95

Ala Glu Ser Gly His Lys Arg Asn Ile Ile Ile Phe His Cys Glu Phe
            100                 105                 110

Ser Ser Glu Arg Gly Pro Lys Met Ser Arg Gly Leu Arg Asn Leu Asp
            115                 120                 125

Arg Glu Arg Asn Thr Asn Ala Tyr Pro Ala Leu His Tyr Pro Glu Ile
        130                 135                 140

Tyr Leu Leu His Asn Gly Tyr Lys Glu Phe Phe Glu Ser His Val Glu
145                 150                 155                 160

Leu Cys Glu Pro His Ala Tyr Arg Thr Met Leu Asp Pro Ala Tyr Asn
                165                 170                 175

Glu Ala Tyr Arg His Phe Arg Ala Lys Ser Lys Ser Xaa Trp Asn Gly
            180                 185                 190

Asp Gly Leu Gly Gly Ala Thr Gly Arg Leu Lys Lys Ser Arg Ser Arg
            195                 200                 205

Leu Met Leu
    210
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 211 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Ser Thr Lys Glu Ser Glu Arg Phe Ile Ser Ser His Val Glu Asp Leu
1               5                   10                  15

Ser Leu Pro Cys Phe Ala Val Lys Glu Asp Ser Leu Lys Arg Ile Thr
            20                  25                  30

Gln Glu Thr Leu Leu Gly Leu Leu Asp Gly Lys Phe Lys Asp Ile Phe
            35                  40                  45

Asp Lys Cys Ile Ile Ile Asp Cys Arg Phe Glu Tyr Glu Tyr Leu Gly
    50                  55                  60

Gly His Ile Ser Thr Ala Val Asn Leu Asn Thr Lys Gln Ala Ile Val
65                  70                  75                  80

Asp Ala Phe Leu Ser Lys Pro Leu Thr Xaa Xaa Xaa Xaa Xaa Xaa Xaa
                85                  90                  95

Xaa Xaa Xaa Xaa His Val Arg Ala Xaa Leu Val Phe His Cys Glu His
            100                 105                 110

Ser Ala His Arg Ala Pro His Leu Ala Leu His Phe Arg Asn Thr Asp
        115                 120                 125

Arg Arg Met Asn Ser His Arg Tyr Pro Phe Leu Tyr Tyr Pro Glu Val
    130                 135                 140

Tyr Ile Leu His Gly Gly Tyr Lys Ser Phe Tyr Glu Asn His Lys Asn
145                 150                 155                 160

Arg Cys Asp Pro Ile Asn Tyr Val Pro Met Asn Asp Arg Ser His Val
            165                 170                 175

Asn Thr Cys Thr Lys Ala Met Asn Asn Phe Lys Arg Xaa Asn Ala Thr
            180                 185                 190

Phe Met Arg Thr Lys Ser Tyr Thr Phe Trp Pro Lys Cys Val Ser Phe
        195                 200                 205

Pro Arg Arg
    210
```

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 75 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
        Thr Asp Gly Lys Arg Val Ile Val Val Phe His Cys Glu Phe Ser Ser
        1               5               10              15

        Glu Arg Gly Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp Arg Leu
                    20              25              30

        Gly Asn Glu Xaa Xaa Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val
                35              40              45

        Leu Lys Gly Gly Tyr Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys
                50              55              60

        Glu Pro Pro Ser Tyr Arg Pro Met His His Glu
        65              70              75
```

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: .peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
        Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser Ser
        1               5               10              15

        Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg Ala
                    20              25              30

        Val Asn Asp Xaa Xaa Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile
                35              40              45

        Leu Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys
                50              55              60

        Glu Pro Gln Asp Tyr Arg Pro Met Asn His Glu
        65              70              75
```

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
        Asp Thr Gln Lys Arg Ile Ile Ile Val Phe His Cys Glu Phe Ser Ser
        1               5               10              15

        Glu Arg Gly Pro Arg Met Cys Arg Cys Leu Arg Glu Glu Asp Arg Ser
                    20              25              30
```

```
        Leu Asn Gln Xaa Xaa Tyr Pro Ala Leu Tyr Tyr Pro Glu Leu Tyr Ile
             35                  40                  45

        Leu Lys Gly Gly Tyr Arg Asp Phe Phe Pro Glu Tyr Met Glu Leu Cys
             50                  55                  60

        Glu Pro Gln Ser Tyr Cys Pro Met His His Gln
             65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
        Ser Gly His Lys Arg Asn Ile Ile Ile Phe His Cys Glu Phe Ser Ser
        1             5                  10                  15

        Glu Arg Gly Pro Lys Met Ser Arg Gly Leu Arg Asn Leu Asp Arg Glu
                      20                  25                  30

        Arg Asn Thr Asn Ala Tyr Pro Ala Leu His Tyr Pro Glu Ile Tyr Leu
                 35                  40                  45

        Leu His Asn Gly Tyr Lys Glu Phe Phe Glu Ser His Val Glu Leu Cys
             50                  55                  60

        Glu Pro His Ala Tyr Arg Thr Met Leu Asp Pro
             65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
        Xaa Xaa His Val Arg Ala Xaa Leu Val Phe His Cys Glu His Ser Ala
        1             5                  10                  15

        His Arg Ala Pro His Leu Ala Leu His Phe Arg Asn Thr Asp Arg Arg
                      20                  25                  30

        Met Asn Ser His Arg Tyr Pro Phe Leu Tyr Tyr Pro Glu Val Tyr Ile
                 35                  40                  45
```

```
      Leu His Gly Gly Tyr Lys Ser Phe Tyr Glu Asn His Lys Asn Arg Cys
           50              55              60

      Asp Pro Ile Asn Tyr Val Pro Met Asn Asp Arg
           65              70              75
```

(2) INFORMATION FOR SEQ ID NO:15:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 75 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
      Xaa Xaa Xaa Xaa Asn Glu Pro Val Leu Val His Cys Ala Ala Gly Val
      1               5               10              15

      Asn Arg Ser Gly Ala Met Ile Leu Ala Xaa Xaa Xaa Xaa Tyr Leu Met
               20              25              30

      Ser Lys Asn Lys Glu Ser Leu Pro Met Leu Tyr Phe Leu Tyr Val Tyr
               35              40              45

      His Ser Met Arg Asp Leu Arg Xaa Gly Ala Phe Val Glu Asn Pro Ser
           50              ·55              60

      Phe Lys Arg Xaa Xaa Xaa Xaa Gln Ile Ile Glu
      65              70              75
```

(2) INFORMATION FOR SEQ ID NO:16:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 75 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
      Leu Ser Pro Glu Asn Gly Pro Ile Val Val His Cys Ser Ala Gly Ile
      1               5               10              15

      Gly Arg Ser Gly Thr Phe Cys Leu Ala Asp Thr Cys Leu Leu Leu Met
               20              25              30

      Asp Lys Arg Lys Asp Pro Ser Ser Val Asp Xaa Ile Lys Lys Val Leu
               35              40              45

      Leu Glu Met Arg Arg Phe Arg Met Gly Xaa Leu Ile Gln Thr Ala Asp
               50              55              60

      Gln Leu Arg Phe Ser Tyr Leu Ala Val Ile Glu
      65              70              75
```

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Leu Ser Pro Glu His Gly Pro Val Val Val His Cys Ser Ala Gly Ile
1               5               10              Cys     15

Gly Arg Ser Gly Thr Phe Cys Leu Ala Asp Thr Cys Leu Leu Leu Met
            20              25              30

Asp Lys Arg Lys Asp Pro Ser Ser Val Asp Xaa Leu Lys Lys Val Leu
        35              40              45

Leu Glu Met Arg Lys Phe Arg Met Gly Xaa Leu Ile Gln Thr Ala Asp
        50              55              60

Gln Leu Arg Phe Ser Tyr Leu Ala Val Ile Glu
65              70              75
```

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Leu Asn Pro Asp His Gly Pro Ala Val Ile His Cys Ser Ala Gly Ile
1               5               10              15

Gly Arg Ser Gly Thr Phe Ser Leu Val Asp Thr Cys Leu Val Leu Met
            20              25              30

Glu Lys Gly Asp Asp Ile Asn Xaa Xaa Xaa Xaa Ile Lys Gln Val Leu
        35              40              45

Leu Asn Met Arg Lys Tyr Arg Met Gly Xaa Leu Ile Gln Thr Pro Asp
        50              55              60

Gln Leu Arg Phe Ser Tyr Met Ala Ile Ile Glu
65              70              75
```

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:

```
                        (A) LENGTH: 75 amino acids
                        (B) TYPE: amino acid
                        (D) TOPOLOGY: linear

        5          (ii) MOLECULE TYPE: peptide

                   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

                   Leu Ala Val Asn Asp Val Asp Ala Glu Asp Gly Ala Asp Pro Asn Leu
        10         1               5               10                  15

                   Cys Ser Glu Tyr Val Lys Asp Ile Tyr Ala Tyr Leu Arg Gln Leu Glu
                                   20                  25                  30

        15         Glu Glu Gln Ala Val Arg Pro Lys Tyr Leu Leu Gly Arg Glu Val Thr
                                   35                  40                  45

                   Gly Asn Met Arg Ala Ile Leu Ile Asp Trp Leu Val Gln Xaa Xaa Val
                                   50                  55                  60
        20
                   Gln Met Lys Phe Arg Leu Leu Gln Xaa Xaa Glu
                   65                  70                  75


        25         (2) INFORMATION FOR SEQ ID NO:20:

                        (i) SEQUENCE CHARACTERISTICS:
                            (A) LENGTH: 75 amino acids
                            (B) TYPE: amino acid
        30              (D) TOPOLOGY: linear

                   (ii) MOLECULE TYPE: peptide

                   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

        35         Ile His Val Lys Asp Val Asp Ala Asp Asp Gly Asn Pro Met Leu
                   1               5               10                  15

                   Cys Ser Glu Tyr Val Lys Asp Ile Tyr Ala Tyr Leu Arg Ser Leu Glu
        40                         20                  25                  30

                   Asp Ala Gln Ala Val Arg Gln Asn Tyr Leu His Gly Gln Glu Val Thr
                                   35                  40                  45

        45         Gly Asn Met Arg Ala Ile Leu Ile Asp Trp Leu Val Gln Xaa Xaa Val
                                   50                  55                  60

                   Gln Met Arg Phe Arg Leu Leu Gln Xaa Xaa Glu
                   65                  70                  75
        50

                   (2) INFORMATION FOR SEQ ID NO:21:

                        (i) SEQUENCE CHARACTERISTICS:
        55              (A) LENGTH: 75 amino acids
                        (B) TYPE: amino acid
                        (D) TOPOLOGY: linear
```

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Thr Ser Val Glu Asp Ile Asp Ala Asp Asp Gly Gly Asn Pro Gln Leu
1               5                   10                  15

Cys Ser Asp Tyr Val Met Asp Ile Tyr Asn Tyr Leu Lys Gln Leu Glu
            20                  25                  30

Val Gln Gln Ser Val His Pro Cys Tyr Leu Glu Gly Lys Glu Ile Asn
            35                  40                  45

Glu Arg Met Arg Ala Ile Leu Val Asp Trp Leu Val Gln Xaa Xaa Val
        50                  55                  60

His Ser Arg Phe Gln Leu Leu Gln Xaa Xaa Glu
65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Leu Cys Cys Glu Val Glu Thr Ile Arg Arg Ala Tyr Pro Asp Ala Asn
1               5                   10                  15

Leu Leu Asn Asp Arg Val Leu Arg Ala Met Leu Lys Ala Glu Glu Thr
            20                  25                  30

Cys Ala Pro Ser Val Ser Tyr Phe Lys Cys Val Gln Lys Glu Val Leu
            35                  40                  45

Pro Ser Met Arg Lys Ile Val Ala Thr Trp Met Leu Glu Xaa Xaa Val
        50                  55                  60

Cys Glu Glu Gln Lys Cys Glu Glu Xaa Xaa Glu
65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Met Ser Ile Val Leu Glu Asp Glu Lys Pro Val Ser Val Asn Glu Val
```

```
     1                    5                        10                       15

        Pro Asp Tyr His Glu Asp Ile His Thr Tyr Leu Arg Glu Met Glu Val
                     20                      25                  30

        Lys Cys Lys Pro Lys Val Gly Tyr Met Lys Lys Gln Pro Asp Ile Thr
                     35                  40                  45

        Asn Ser Met Arg Ala Ile Leu Val Asp Trp Leu Val Glu Xaa Xaa Val
                 50                  55                  60

        Gly Glu Glu Tyr Lys Leu Gln Asn Xaa Xaa Glu
        65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
        Ile Ile Asp Cys Arg Thr Phe Pro Glu Tyr Glu
        1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
        Ala Thr Ile Ala Thr Ile Gly Ala Thr Thr Gly Cys Cys Gly Ile Thr
        1               5                   10                  15

        Ala Thr Cys Cys Cys Ile Thr Ala Cys Thr Gly Ala
                     20                  25
```

(2) INFORMATION FOR SEQ ID NO:26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
        Ala Thr Ile Ala Thr Ile Gly Ala Thr Thr Gly Cys Cys Gly Ile Thr
        1               5                   10                  15

        Ala Thr Cys Gly Ala Ile Thr Ala Cys Thr Gly Ala
                    20                  25
```

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATAGAACTTC AGCAAGTGAG AAAGTA   26

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
        Cys Gln Gly Ala Leu Asn Leu Tyr Ser Gln Glu Glu Leu Phe
        1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:29:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
        Cys Lys Gly Ala Val Asn Leu His Met Glu Glu Glu Val Glu
        1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:30:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Cys Lys Lys Lys Val Glu Lys Ile Gly Glu Gly Thr Tyr Gly Val Val
1   5   10   15

Tyr Lys

(2) INFORMATION FOR SEQ ID NO:31:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Leu Val Phe His Cys Glu Xaa Xaa Xaa Xaa Arg
1   5   10

Claims

1. Purified *CDC25*A protein which is of mammalian origin.

2. The *CDC25*A protein of claim 1, which protein is a product of recombinant expression.

3. Purified *CDC25*B protein which is of mammalian origin.

4. The *CDC25*B protein of claim 3, which protein is a product of recombinant expression.

5. A recombinant *CDC25*A polypeptide comprising an amino acid sequence designated in SEQ ID NO: 2, which recombinant polypeptide possesses an endogenous tyrosine phosphatase activity.

6. The recombinant polypeptide of Claim 5, which recombinant polypeptide is a fusion protein.

7. The recombinant polypeptide of Claim 6, wherein the fusion protein further includes a glutathione-S-transferase amino acid sequence.

8. The recombinant polypeptide of Claim 5, which endogenous tyrosine phosphatase activity hydrolyzes p-nitrophenylphosphate.

9. The recombinant polypeptide of Claim 5, which recombinant polypeptide rescues a mutant *cdc25-22* strain of fission yeast.

10. The recombinant polypeptide of Claim 5, which endogenous tyrosine phosphatase activity dephosphorylates a phosphorylated catalytic subunit of an M-phase kinase.

11. The recombinant polypeptide of Claim 5, which endogenous tyrosine phosphatase activity dephosphorylates a phosphorylated CDC2 kinase.

5   12. A recombinant *CDC25B* polypeptide comprising an amino acid sequence designated in SEQ ID NO: 4, which recombinant polypeptide possesses an endogenous tyrosine phosphatase activity.

10 13. The recombinant polypeptide of Claim 12, which recombinant polypeptide is a fusion protein.

14. The recombinant polypeptide of Claim 13, wherein the fusion protein further includes a glutathione-S-
15 transferase amino acid sequence.

15. The recombinant polypeptide of Claim 12, which recombinant polypeptide hydrolyzes p-nitrophenylphosphate.

20

16. The recombinant polypeptide of Claim 12, which recombinant polypeptide rescues a mutant *cdc25-22* strain of fission yeast.

25 17. The recombinant polypeptide of Claim 12, which endogenous tyrosine phosphatase activity dephosphorylates a phosphorylated catalytic subunit of an M-phase kinase.

18. The recombinant polypeptide of Claim 12, which endogenous
30   tyrosine phosphatase activity dephosphorylates a phosphorylated CDC2 kinase.

19. A recombinant *CDC25A* polypeptide encoded by a nucleic acid comprising a nucleotide sequence which
35   (i) specifically hybridizes under high stringency conditions to the *CDC25A* gene designated by SEQ ID No. 1, and

(ii) encodes a polypeptide which possesses an endogenous catalytic phosphatase activity.

20. The recombinant polypeptide of Claim 19, which recombinant polypeptide is a fusion protein.

21. A recombinant *CDC25B* polypeptide encoded by a nucleic acid comprising a nucleotide sequence which
(i) specifically hybridizes under high stringency conditions to the *CDC25B* gene designated by SEQ ID No. 3, and
(ii) encodes a polypeptide which possesses an endogenous catalytic phosphatase activity.

22. The recombinant polypeptide of Claim 21, which recombinant polypeptide is a fusion protein.

23. A recombinant *CDC25A* polypeptide comprising an amino acid sequence designated in SEQ ID NO: 2, which recombinant polypeptide binds to a phosphotyrosine containing cyclin dependent kinase.

24. The recombinant polypeptide of Claim 23, which cyclin dependent kinase is a CDC2 kinase.

25. A recombinant *CDC25B* polypeptide comprising an amino acid sequence designated in SEQ ID NO: 4, which recombinant polypeptide binds to a phosphotyrosine containing cyclin dependent kinase.

26. The recombinant polypeptide of Claim 25, which cyclin dependent kinase is a CDC2 kinase.

27. A purified or recombinant polypeptide which is immunoprecipitated by an antibody against the *CDC25A* protein designated by SEQ ID NO: 2, which polypeptide binds to a phosphotyrosine containing cyclin dependent kinase.

28. A purified or recombinant polypeptide which is immunoprecipitated by an antibody against the *CDC25A* protein designated by SEQ ID NO: 4, which polypeptide binds to a phosphotyrosine containing cyclin dependent kinase.

29. An antibody which specifically binds a mammalian *CDC25A* protein.

30. An antibody which specifically binds a mammalian *CDC25B* protein.

NOVEL HUMAN cdc25 GENES, ENCODED
PRODUCTS AND USES THEREFOR

Abstract of the Disclosure

Two previously undescribed human cdc25 genes,
5 designated cdc25 A and cdc25 B, which have been shown to
have an endogenous tyrosine phosphatase activity that can
be specifically activated by B-type cyclin, in the
complete absence of cdc2 are described. As a result of
this work, new approaches to regulating the cell cycle in
10 eukaryotic cells and, particularly, to regulating the
activity of tyrosine specific phosphatases which play a
key role in the cell cycle are available. Applicant's
invention relates to methods of regulating the cell cycle
and, specifically, to regulating activation of
15 cdc2-kinase, through alteration of the activity and/or
levels of tyrosine phosphatases or through alteration of
the interaction of components of MPF. The present
invention also relates to agents or compositions useful in
the method of regulating (inhibiting or enhancing) the
20 cell cycle. Such agents or compositions can be inhibitors
(such as low molecular weight peptides or compounds,
either organic or inorganic) of the catalytic activity of
tyrosine specific PTPases (particularly cdc25), blocking
agents which interfere with interaction or binding of the
25 tyrosine specific PTPase with cyclin or the cyclin/cdc2
complex, or agents which interfere directly with the
catalytic activity of the PTPases. The invention also
pertains to an assay for identifying agents which after
stimulation of kinase activity of pre-MPF and thus alter
30 activation of MPF and entry into mitosis. Such agents are
also the subject of this invention.

                                             Met Glu Leu Gly Pro
                                              1   5

AGC CCC GCA CCG CGC CGC CTG CTC TTC GCC TGC AGC CCC CCT CCC GCG   522
Ser Pro Ala Pro Arg Arg Leu Leu Phe Ala Cys Ser Pro Pro Pro Ala
              10   15   20

TCG CAG CCC GTC GTG AAG GCG CTA TTT GGC GCT TCA GCC GCC GGG GGA   570
Ser Gln Pro Val Val Lys Ala Leu Phe Gly Ala Ser Ala Ala Gly Gly
          25   30   35

CTG TCG CCT GTC ACC AAC CTG ACC GTC ACT ATG GAC CAG CTG CAG GGT   618
Leu Ser Pro Val Thr Asn Leu Thr Val Thr Met Asp Gln Leu Gln Gly
          40   45   50

CTG GGC AGT GAT TAT GAG CAA CCA CTG GAG GTG AAG AAC AAC AGT AAT   666
Leu Gly Ser Asp Tyr Glu Gln Pro Leu Glu Val Lys Asn Asn Ser Asn
     55   60   65

CTG CAG AGA ATG GGC TCC TCC GAG TCA ACA GAT TCA GGT TTC TGT CTA   714
Leu Gln Arg Met Gly Ser Ser Glu Ser Thr Asp Ser Gly Phe Cys Leu
70   75   80   85

GAT TCT CCT GGG CCA TTG GAC AGT AAA GAA AAC CTT GAA AAT CCT ATG   762
Asp Ser Pro Gly Pro Leu Asp Ser Lys Glu Asn Leu Glu Asn Pro Met
              90   95   100

AGA AGA ATA CAT TCC CTA CCT CAA AAG CTG TTG GGA TGT AGT CCA GCT   810
Arg Arg Ile His Ser Leu Pro Gln Lys Leu Leu Gly Cys Ser Pro Ala
              105   110   115

CTG AAG AGG AGC CAT TCT GAT TCT CTT GAC CAT GAC ATC TTT CAG CTC   858
Leu Lys Arg Ser His Ser Asp Ser Leu Asp His Asp Ile Phe Gln Leu
              120   125   130

ATC GAC CCA GAT GAG AAC AAG GAA AAT GAA GCC TTT GAG TTT AAG AAG   906
Ile Asp Pro Asp Glu Asn Lys Glu Asn Glu Ala Phe Glu Phe Lys Lys
     135   140   145

CCA GTA AGA CCT GTA TCT CGT GGC TGC CTG CAC TCT CAT GGA CTC CAG   954
Pro Val Arg Pro Val Ser Arg Gly Cys Leu His Ser His Gly Leu Gln
150   155   160   165

CAG GGT AAA GAT CTC TTC ACA CAG AGG CAG AAC TCT GCC CAG CTC GGA   1002
Glu Gly Lys Asp Leu Phe Thr Gln Arg Gln Asn Ser Ala Gln Leu Gly
              170   175   180

Figure 1(a) - Panel A

```
ATG CTT TCC TCA AAT GAA AGA GAT AGC AGT GAA CCA GGG AAT TTC ATT          1050
Met Leu Ser Ser Asn Glu Arg Asp Ser Ser Glu Pro Gly Asn Phe Ile
        185                     190                     195

CCT CTT TTT ACA CCC CAG TCA CCT GTG ACA GCC ACT TTG TCT GAT GAG          1098
Pro Leu Phe Thr Pro Gln Ser Pro Val Thr Ala Thr Leu Ser Asp Glu
        200                     205                     210

GAT GAT GGC TTC GTG GAC CTT CTC GAT GGA GAG AAT CTG AAG AAT GAG          1146
Asp Asp Gly Phe Val Asp Leu Leu Asp Gly Glu Asn Leu Lys Asn Glu
        215                     220                     225

GAG GAG ACC CCC TCG TGC ATG GCA AGC CTC TGG ACA GCT CCT CTC GTC          1194
Glu Glu Thr Pro Ser Cys Met Ala Ser Leu Trp Thr Ala Pro Leu Val
230                     235                     240                 245

ATG AGA ACT ACA AAC CTT GAC AAC CGA TGC AAG CTG TTT GAC TCC CCT          1242
Met Arg Thr Thr Asn Leu Asp Asn Arg Cys Lys Leu Phe Asp Ser Pro
                    250             255                 260

TCC CTG TGT AGC TCC AGC ACT CGG TCA GTG TTG AAG AGA CCA GAA CGT          1290
Ser Leu Cys Ser Ser Ser Thr Arg Ser Val Leu Lys Arg Pro Glu Arg
                265                 270                 275

TCT CAA GAG GAG TCT CCA CCT GGA AGT ACA AAG AGG AGG AAG AGC ATG          1338
Ser Gln Glu Glu Ser Pro Pro Gly Ser Thr Lys Arg Arg Lys Ser Met
                280                 285                 290

TCT GGG GCC AGC CCC AAA GAG TCA ACT AAT CCA GAG AAG GCC CAT GAG          1386
Ser Gly Ala Ser Pro Lys Glu Ser Thr Asn Pro Glu Lys Ala His Glu
        295                 300                 305

ACT CTT CAT CAG TCT TTA TCC CTG GCA TCT TCC CCC AAA GGA ACC ATT          1434
Thr Leu His Gln Ser Leu Ser Leu Ala Ser Ser Pro Lys Gly Thr Ile
310                     315                 320                 325

GAG AAC ATT TTG GAC AAT GAC CCA AGG GAC CTT ATA GGA GAC TTC TCC          1482
Glu Asn Ile Leu Asp Asn Asp Pro Arg Asp Leu Ile Gly Asp Phe Ser
                330                 335                 340

AAG GGT TAT CTC TTT CAT ACA GTT GCT GGG AAA CAT CAG GAT TTA AAA          1530
Lys Gly Tyr Leu Phe His Thr Val Ala Gly Lys His Gln Asp Leu Lys
                345                 350                 355

TAC ATC TCT CCA GAA ATT ATG GCA TCT GTT TTG AAT GGC AAG TTT GCC          1578
Tyr Ile Ser Pro Glu Ile Met Ala Ser Val Leu Asn Gly Lys Phe Ala
                360                 365                 370

AAC CTC ATT AAA GAG TTT GTT ATC ATC GAC TGT CGA TAC CCA TAT GAA          1626
Asn Leu Ile Lys Glu Phe Val Ile Ile Asp Cys Arg Tyr Pro Tyr Glu
        375                 380                 385

TAC GAG GGA GGC CAC ATC AAG GGT GCA GTG AAC TTG CAC ATG GAA GAA          1674
Tyr Glu Gly Gly His Ile Lys Gly Ala Val Asn Leu His Met Glu Glu
390                 395                 400                     405

GAG GTT GAA GAC TTC TTA TTG AAG AAG CCC ATT GTA CCT ACT GAT GGC          1722
Glu Val Glu Asp Phe Leu Leu Lys Lys Pro Ile Val Pro Thr Asp Gly
                410                 415                 420
```

Figure 1(b) - Panel A

AAG CGT GTC ATT GTT GTG TTT CAC TGC GAG TTT TCT TCT GAG AGA GGT   1770
Lys Arg Val Ile Val Val Phe His Cys Glu Phe Ser Ser Glu Arg Gly
            425   430   435

CCC CGC ATG TGC CGG TAT GTG AGA GAG AGA GAT CGC CTG GGT AAT GAA   1818
Pro Arg Met Cys Arg Tyr Val Arg Glu Arg Asp Arg Leu Gly Asn Glu
            440   445   450

TAC CCC AAA CTC CAC TAC CCT GAG CTG TAT GTC CTG AAG GGG GGA TAC   1866
Tyr Pro Lys Leu His Tyr Pro Glu Leu Tyr Val Leu Lys Gly Gly Tyr
            455   460   465

AAG GAG TTC TTT ATG AAA TGC CAG TCT TAC TGT GAG CCC CCT AGC TAC   1914
Lys Glu Phe Phe Met Lys Cys Gln Ser Tyr Cys Glu Pro Pro Ser Tyr
470   475   480   485

CGG CCC ATG CAC CAC GAG GAC TTT AAA GAA GAC CTG AAG AAG TTC CGC   1962
Arg Pro Met His His Glu Asp Phe Lys Glu Asp Leu Lys Lys Phe Arg
            490   495   500

ACC AAG AGC CGG ACC TGG GCA GGG GAG AAG AGC AAG AGG GAG ATG TAC   2010
Thr Lys Ser Arg Thr Trp Ala Gly Glu Lys Ser Lys Arg Glu Met Tyr
            505   510   515

AGT CGT CTG AAG AAG CTC TGAGGGCGGC AGGACCAGCC AGCAGCAGCC   2058
Ser Arg Leu Lys Lys Leu
            520

CAAGCTTCCC TCCATCCCCC TTTACCCTCT TTCCTGCAGA GAAACTTAAG CAAAGGGGAC   2118

AGCTGTGTGA CATTTGGAGA GGGGGCCTGG GACTTCCATG CCTTAAACCT ACCTCCCACA   2178

CTCCCAAGGT TGGAGCCCAG GGCATCTTGC TGGCTACGCC TCTTCTGTCC CTGTTAGACG   2238

TCCTCCGTCC ATATCAGAAC TGTGCCACAA TGCAGTTCTG AGCACCGTGT CAAGCTGCTC   2298

TCAGCCACAG TGGGATGAAC CAGCCGGGGC CTTATCGGGC TCCAGCATCT CATGAGGGGA   2358

CAGGAGACGG AGGGGAGTAG AGAAGTTTAC ACAGAAATGC TGCTGGCCAA ATAGCAAAGA   2418

G   2419

Figure 1(c) - Panel A

```
CTGCCCTGCG CCCGGCCCTC CAGCCAGCCT GCCAGCTGTG CCGGCGTTTG TTGGTCTGCC          60

CGCCCCGCCG CG ATG GAG GTG CCC CAG CCG GAG CCC GCG CCA GGC TCG              108
              Met Glu Val Pro Gln Pro Glu Pro Ala Pro Gly Ser
               1                   5                      10

GCT CTC AGT CCA GCA GGC GTG TGC GGT GGC GCC CAG CGT CCG GGC CAC          156
Ala Leu Ser Pro Ala Gly Val Cys Gly Gly Ala Gln Arg Pro Gly His
             15                  20                  25

CTC CCG GGC CTC CTG CTG GGA TCT CAT GGC CTC CTG GGG TCC CCG GTG          204
Leu Pro Gly Leu Leu Leu Gly Ser His Gly Leu Leu Gly Ser Pro Val
         30                  35                  40

CGG GCG GCC GCT TCC TCG CCG GTC ACC ACC CTC ACC CAG ACC ATG CAC          252
Arg Ala Ala Ala Ser Ser Pro Val Thr Thr Leu Thr Gln Thr Met His
45                  50                  55                  60

GAC CTC GCC GGG CTC GGC AGC CGC AGC CGC CTG ACG CAC CTA TCC CTG          300
Asp Leu Ala Gly Leu Gly Ser Arg Ser Arg Leu Thr His Leu Ser Leu
                 65                  70                  75

TCT CGA CGG GCA TCC GAA TCC TCC CTG TCG TCT GAA TCC TCC GAA TCT          348
Ser Arg Arg Ala Ser Glu Ser Ser Leu Ser Ser Glu Ser Ser Glu Ser
                 80                  85                  90

TCT GAT GCA GGT CTC TGC ATG GAT TCC CCC AGC CCT ATG GAC CCC CAC          396
Ser Asp Ala Gly Leu Cys Met Asp Ser Pro Ser Pro Met Asp Pro His
             95                  100                 105

ATG GCG GAG CAG ACG TTT GAA CAG GCC ATC CAG GCA GCC AGC CGG ATC          444
Met Ala Glu Gln Thr Phe Glu Gln Ala Ile Gln Ala Ala Ser Arg Ile
     110                 115                 120

ATT CGA AAC GAG CAG TTT GCC ATC AGA CGC TTC CAG TCT ATG CCG GTG          492
Ile Arg Asn Glu Gln Phe Ala Ile Arg Arg Phe Gln Ser Met Pro Val
125                 130                 135                 140

AGG CTG CTG GGC CAC AGC CCC GTG CTT CGG AAC ATC ACC AAC TCC CAG          540
Arg Leu Leu Gly His Ser Pro Val Leu Arg Asn Ile Thr Asn Ser Gln
                 145                 150                 155

GCG CCC GAC GGC CGG AGG AAG AGC GAG GCG GGC AGT GGA GCT GCC AGC          588
Ala Pro Asp Gly Arg Arg Lys Ser Glu Ala Gly Ser Gly Ala Ala Ser
                 160                 165                 170

AGC TCT GGG GAA GAC AAG GAG AAT GAT GGA TTT GTC TTC AAG ATG CCA          636
Ser Ser Gly Glu Asp Lys Glu Asn Asp Gly Phe Val Phe Lys Met Pro
         175                 180                 185

TGG AAG CCC ACA CAT CCC AGC TCC ACC CAT GCT CTG GCA GAG TGG GCC          684
Trp Lys Pro Thr His Pro Ser Ser Thr His Ala Leu Ala Glu Trp Ala
         190                 195                 200

AGC CGC AGG GAA GCC TTT GCC CAG AGA CCC AGC TCG GCC CCC GAC CTG          732
Ser Arg Arg Glu Ala Phe Ala Gln Arg Pro Ser Ser Ala Pro Asp Leu
205                 210                 215                 220

ATG TGT CTC AGT CCT GAC CGG AAG ATG GAA GTG GAG GAG CTC AGC CCC          780
Met Cys Leu Ser Pro Asp Arg Lys Met Glu Val Glu Glu Leu Ser Pro
                 225                 230                 235
```

Figure 1(d) - Panel B

```
CTG GCC CTA GGT CGC TTC TCT CTG ACC CCT GCA GAG GGG GAT ACT GAG      828
Leu Ala Leu Gly Arg Phe Ser Leu Thr Pro Ala Glu Gly Asp Thr Glu
        240             245                 250

GAA GAT GAT GGA TTT GTG GAC ATC CTA GAG AGT GAC TTA AAG GAT GAT      876
Glu Asp Asp Gly Phe Val Asp Ile Leu Glu Ser Asp Leu Lys Asp Asp
        255             260                 265

GAT GCA GTT CCC CCA GGC ATG GAG AGT CTC ATT AGT GCC CCA CTG GTC      924
Asp Ala Val Pro Pro Gly Met Glu Ser Leu Ile Ser Ala Pro Leu Val
        270             275                 280

AAG ACC TTG GAA AAG GAA GAG GAA AAG GAC CTC GTC ATG TAC AGC AAG      972
Lys Thr Leu Glu Lys Glu Glu Glu Lys Asp Leu Val Met Tyr Ser Lys
285             290                 295                 300

TGC CAG CGG CTC TTC CGC TCT CCG TCC ATG CCC TGC AGC GTG ATC CGG     1020
Cys Gln Arg Leu Phe Arg Ser Pro Ser Met Pro Cys Ser Val Ile Arg
                305                 310                 315

CCC ATC CTC AAG AGG CTG GAG CGG CCC CAG GAC AGG GAC ACG CCC GTG     1068
Pro Ile Leu Lys Arg Leu Glu Arg Pro Gln Asp Arg Asp Thr Pro Val
                320                 325                 330

CAG AAT AAG CGG AGG CGG AGC GTG ACC CCT CCT GAG GAG CAG CAG GAG     1116
Gln Asn Lys Arg Arg Arg Ser Val Thr Pro Pro Glu Glu Gln Gln Glu
                335                 340                 345

GCT GAG GAA CCT AAA GCC CGC GTC CTC CGC TCA AAA TCA CTG TGT CAC     1164
Ala Glu Glu Pro Lys Ala Arg Val Leu Arg Ser Lys Ser Leu Cys His
350             355                 360

GAT GAG ATC GAG AAC CTC CTG GAC AGT GAC CAC CGA GAG CTG ATT GGA     1212
Asp Glu Ile Glu Asn Leu Leu Asp Ser Asp His Arg Glu Leu Ile Gly
365             370                 375                 380

GAT TAC TCT AAG GCC TTC CTC CTA CAG ACA GTA GAC GGA AAG CAC CAA     1260
Asp Tyr Ser Lys Ala Phe Leu Leu Gln Thr Val Asp Gly Lys His Gln
                385                 390                 395

GAC CTC AAG TAC ATC TCA CCA GAA ACG ATG GTG GCC CTA TTG ACG GGC     1308
Asp Leu Lys Tyr Ile Ser Pro Glu Thr Met Val Ala Leu Leu Thr Gly
                400                 405                 410

AAG TTC AGC AAC ATC GTG GAT AAG TTT GTG ATT GTA GAC TGC AGA TAC     1356
Lys Phe Ser Asn Ile Val Asp Lys Phe Val Ile Val Asp Cys Arg Tyr
                415                 420                 425

CCC TAT GAA TAT GAA GGC GGG CAC ATC AAG ACT GCG GTG AAC TTG CCC     1404
Pro Tyr Glu Tyr Glu Gly Gly His Ile Lys Thr Ala Val Asn Leu Pro
                430                 435                 440

CTG GAA CGC GAC GCC GAG AGC TTC CTA CTG AAG AGC CCC ATC GCG CCC     1452
Leu Glu Arg Asp Ala Glu Ser Phe Leu Leu Lys Ser Pro Ile Ala Pro
445             450                 455                 460

TGT AGC CTG GAC AAG AGA GTC ATC CTC ATT TTC CAC TGT GAA TTC TCA     1500
Cys Ser Leu Asp Lys Arg Val Ile Leu Ile Phe His Cys Glu Phe Ser
                465                 470                 475
```

Figure 1(e) - Panel B

```
TCT GAG CGT GGG CCC CGC ATG TGC CGT TTC ATC AGG GAA CGA GAC CGT        1548
Ser Glu Arg Gly Pro Arg Met Cys Arg Phe Ile Arg Glu Arg Asp Arg
            480                 485                 490

GCT GTC AAC GAC TAC CCC AGC CTC TAC TAC CCT GAG ATG TAT ATC CTG        1596
Ala Val Asn Asp Tyr Pro Ser Leu Tyr Tyr Pro Glu Met Tyr Ile Leu
            495                 500                 505

AAA GGC GGC TAC AAG GAG TTC TTC CCT CAG CAC CCG AAC TTC TGT GAA        1644
Lys Gly Gly Tyr Lys Glu Phe Phe Pro Gln His Pro Asn Phe Cys Glu
    510                 515                 520

CCC CAG GAC TAC CGG CCC ATG AAC CAC GAG GCC TTC AAG GAT GAG CTA        1692
Pro Gln Asp Tyr Arg Pro Met Asn His Glu Ala Phe Lys Asp Glu Leu
525                 530                 535                 540

AAG ACC TTC CGC CTC AAG ACT CGC AGC TGG GCT GGG GAG CGG AGC CGG        1740
Lys Thr Phe Arg Leu Lys Thr Arg Ser Trp Ala Gly Glu Arg Ser Arg
            545                 550                 555

CGG GAG CTC TGT AGC CGG CTG CAG GAC CAG TGAGGGCCT GCCCCAGTCC           1790
Arg Glu Leu Cys Ser Arg Leu Gln Asp Gln
            560                 565

TGCTACCTCC CTTGCCTTTC GAGGCCTGAA GCCAGCTGCC CTATGGGCCT GCCGGGCTGA      1850

GGGCCTGCTG GAGGCCTCAG GTGCTGTCCA TGGGAAAGAT GGTGTGGTGT CCTGCCTGTC      1910

TGCCCCAGCC CAGATTCCCC TGTGTCATCC CATCATTTTC CATATCCTGG TGCCCCCCAC      1970

CCCTGGAAGA GCCCAGTCTG TTGAGTTAGT TAAGTTGGGT TAATACCAGC TTAAAGGCAG      2030

TATTTTCTGT CCTCCAGGAG CTTCTTGTTT CCTTGTTAGG GTTAACCCTT CATCTTCCTG      2090

TGTCCTGAAA CGCTCCTTTG TGTGTGTGTC AGCTGAGGCT GGGGAGAGCC GTGGTCCCTG      2150

AGGATGGGTC AGAGCTAAAC TCCTTCCTGG CCTGAGAGTC AGCTCTCTGC CCTGTGTACT      2210

TCCCGGGCCA GGGCTGCCCC TAATCTCTGT AGGAACCGTG GTATGTCTGC CATGTTGCCC      2270

CTTTCTCTTT TCCCCTTTCC TGTCCCACCA TACGAGCACC TCCAGCCTGA ACAGAAGCTC      2330

TTACTCTTTC CTATTTCAGT GTTACCTGTG TGCTTGGTCT GTTTGACTTT ACGCCCATCT      2390

CAGGACACTT CCGTAGACTG TTTAGGTTCC CCTGTCAAAT ATCAGTTACC CACTCGGTCC      2450

CAGTTTTGTT GCCCCAGAAA GGGATGTTAT TATCCTTGGG GGCTCCCAGG CCAAGGGTTA      2510

AGGCCTGAAT CATGAGCCTG CTGGAAGCCC AGCCCCTACT GCTGTGAACC CTGGGGCCTG      2570

ACTGCTCAGA ACTTGCTGCT GTCTTGTTGC CGATGGATGG AAGGTTGGAT GGATGGGTGG      2630

ATGGCCGTGG ATGGCCGTGG ATGCGCAGTG CCTTGCATAC CCAAACCAGG TGGCAGCCTT      2690

TTGTTGAGCA TGACACCTGC AGCAGGAATA TATGTGTGCC TATTTGTGTG GACAAAAATA      2750

TTTACACTTA GGGTTTGGAG CTATTCAAGA GGAAATGTCA CAGAAGCAGC TAAACCAAGG      2810

ACTGAGCACC CTCTGGATTC TGAATCTCAA GATGGGGGCA GGGCTGTGCT TGAAGGCCCT      2870

GCTGAGTCAT CTGTTAGGGC CTTGGTTCAA TAAAGCACTG AGCAAGTTGA GAAAAAAAAA      2930

AAAAAAAAAA                                                             2940
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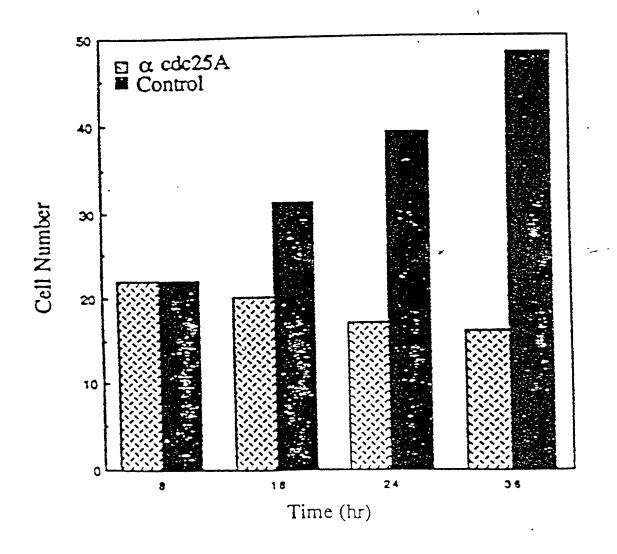
Figure I(f) - Panel B

Block 1:

```
cdc25A  1-318   LDNDPRDLIGDESKGYLFHTVAGKHQDLKYISPEIMASVIVNGKEANIJKEFVIIDCRYPYEYEGGHIKGAVNLHMEEE
cdc25B  1-361   LDSDHRELIGDYSKAFLLQTVDGKHQDLKYISPETMALIIGKEESNIVDCVVIDCRYPYEYEGGHIKTAVNLPLERD
cdc25C  1-276   EDSNQGHLIGDFSKVCAUPTVSGKHQDLKYVNPETVAILLSGKEQGLITEKIYVIDCRYPYEYLGGHIQGALNLYSQEE
stg     1-269   ENRNEPELIGDESKAYSLDLMERTHPDIRSIESEIVARGIAKGESDKVASYRFIIDCRYPYEEGGHIEGAKNIYTTEQ
25Sp    1-362   STKESERFISSHIVEDLSLPCFAVKEDSLKRITQETILGDKEKDIFDKCILIDCRFEYBYLGGHISTAVNLNTKQA
```

Block 2:

```
cdc25A          VEDFLLKKPIVE----------TDGKRVIVMFICEESSSRGPRMCRVVFEHDRLGNE----YPMLHYPELYMLKGGYKEFEM
cdc25B          AESFLLKSPIFEC----------SLDKVVILIFHGEESSERGERGPRMCRFIRERDRVVND----YPSDYPEMYILKGGYKEFFP
cdc25C          LFNEELKKQRIVEL----------DTQKHIIYFHGEESTERGPRMCRCIRERDRSLNQ----YPALVYPELYILNGYKEFFF
stg             ILDEELLTVQQTELQQQQNAESGHKENILLHCEESSSRGPRSGIRNLDRERNTNAYPALHYPELYLLHNGYKEFFE
25Sp            IVDAELISKELT----------HVELA-LVEIICEDISANIIPAHILALHFUNIDIRMNSHRYPFLYYPEVYILIICGGYKSFYE
```

Block 3:

```
cdc25A          KCQSYGEPPSNRPMHREDFKEDLKKTRSRTWAGEKSKREMYSRIKTL   523
cdc25B          QPNFCEPQDYRPMNHEAFKDELKTNERLKTRSWAGERSRRELQSRLQDQ   566
cdc25C          EYMELCEPQSYCPMHHQDHKTELLRCHSQSRVQEGERQLREDIALLVDMSP   474
stg             SHVELCEPHAYRTMLDPAYNEAYRHFPAKSHS-WNGDGLGGATGHLKGSRSRLML   479
25Sp            NAKNFQDPINIVPFMADRSHVNTCTKAMNNFKR-NATFMRTKSYTFWPCVSFPRR   580
```

Figure 2

Figure 3(a)

Figure 3(b)

Figure 4(a)

Figure 4(b)

Figure 4(c)

Figure 5

Figure 6

Figure 7(a)

Figure 7(b)

Figure 8

p34^{cdc2}-p72^{cdc25}association (□—□)
(arbitrary units)

1000 800 600 400 200 0

Time (min)

0   25   50   75   100   125

HI kinase activity (■—■)
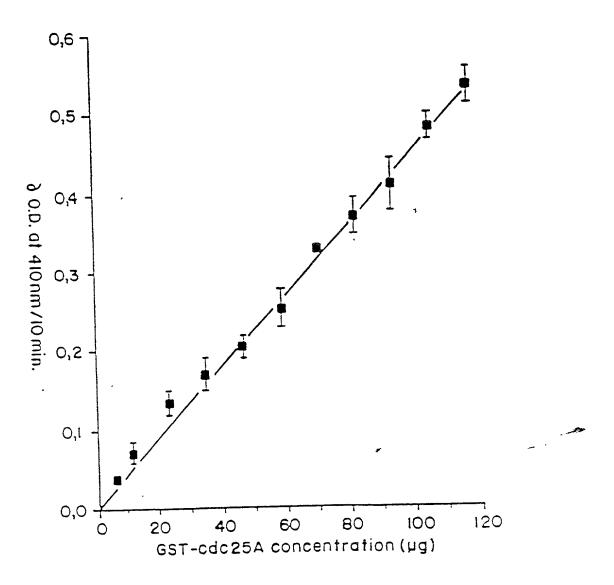(pmol P/mg/min)
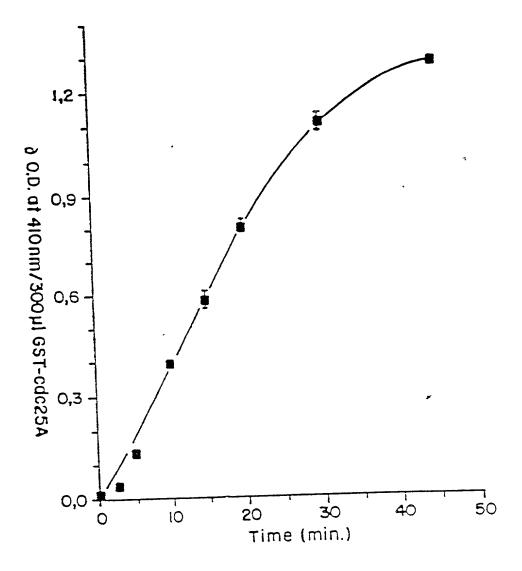
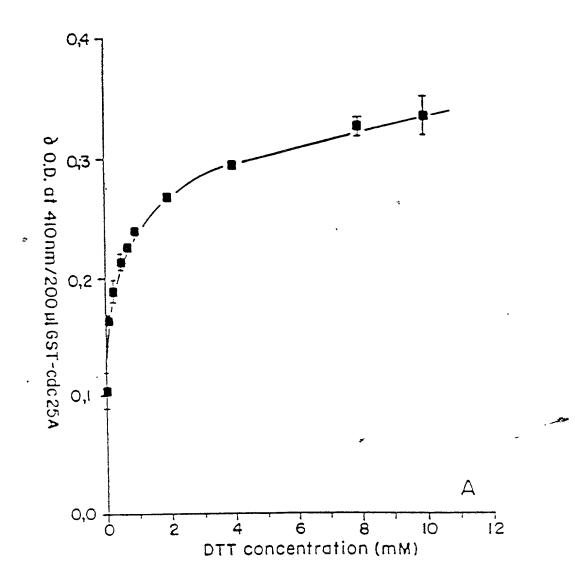1000 800 600 400 200 0
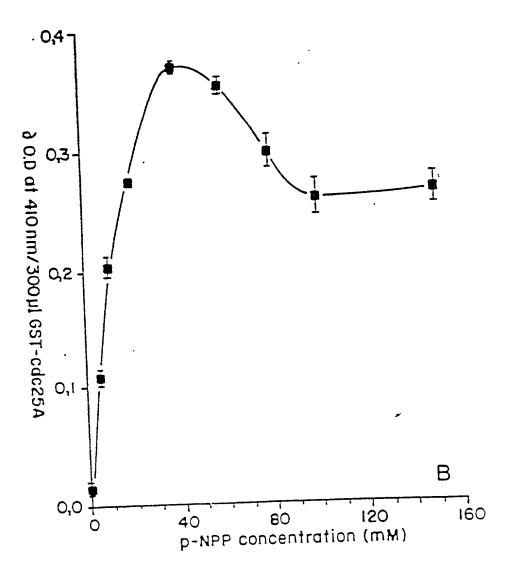
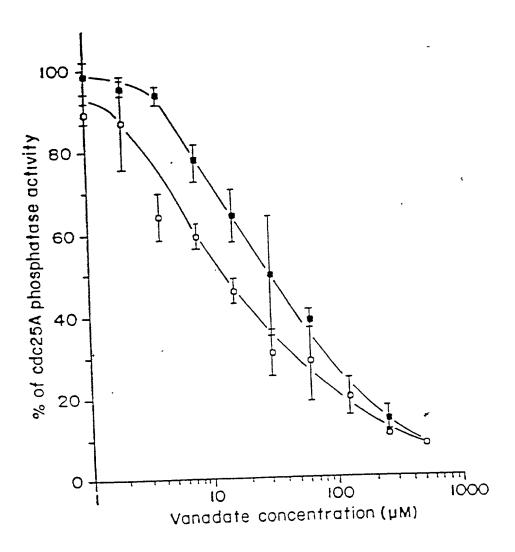Figure 9

Figure 10

Figure 11

Figure 12(a)

Figure 12(b)

Figure 13(a)

Figure 13(b)

Figure 14

Declaration, Petition and Power of Attorney For Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

"NOVEL CDC25 GENES, ENCODED PRODUCTS AND USES THEREOF"

the specification of which was filed on April 24, 1995 in the United States Patent and Trademark Office as U.S.S.N. 08/428,415, which is a continuation-in-part of U.S.S.N. 08/379,685 filed January 26, 1995 and entitled *"NOVEL HUMAN CDC25 GENES, ENCODED PRODUCTS AND USES THEREFOR"* which is a continuation-in-part of U.S.S.N. 08/124,569, filed 20 September 1993, which is a continuation-in-part of U.S.S.N. 07/793,601, filed 18 November 1991, and is a continuation-in-part of U.S.S.N. 08/189,206, filed 31 January 1994, which is a continuation-in-part of U.S.S.N. 07/878,640, filed 5 May 1992, and is a continuation-in-part of U.S.S.N. 07/793,601, filed 18 November 1991.

I do not know and do not believe that the subject matter of this application was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to the date of this application, and that said subject matter has not been patented or made the subject of an issued inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months prior to the date of this application; that I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application, that no application for patent or inventor's certificate on the subject matter of this application has been filed by me or my representatives or assigns in any country foreign to the United States, except those identified below, and that I have reviewed and understand the contents of the specification, including the claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Check one:

    _ no such applications have been filed.

    _X_ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 35 USC 119
PCT	PCT/US92/10052	November 17, 1992	_ Yes No _X_
			_ Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No _

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

-2-

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application. As to subject matter of this application which is common to my earlier United States application, if any, described below, I do not believe that the same was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to said earlier application, or in public use or on sale in the United States more than one year prior to said earlier application, that the said common subject matter has not been patented or made the subject of an inventor's certificate issued before the date of said earlier application in any country foreign to the United States on an application, filed by me or my legal representatives or assigns more than twelve months prior to said application and that no application for patent or inventor's certificate on said subject matter has been filed by me or my representatives or assigns in any country foreign to the United States except those identified herein.

07/793,601	18 November 1991	Abandoned
(Application Serial No.)	(Filing Date)	(Status)

07/878,640	5 May 1992	Issued, U.S.S.N. 5,294,538
(Application Serial No.)	(Filing Date)	(Status)

08/124,569	20 September 1993	*September* ~~Pending~~
(Application Serial No.)	(Filing Date)	(Status)

08/189,206	31 January 1994	Pending
(Application Serial No.)	(Filing Date)	(Status)

08/379,685	26 January 1995	Pending
(Application Serial No.)	(Filing Date)	(Status)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

John A. Lahive, Jr.	Reg. No. 19,788	John V. Bianco	Reg. No. 36,748
W. Hugo Liepmann	Reg. No. 20,407	Jeremiah Lynch	Reg. No. 17,425
James E. Cockfield	Reg. No. 19,162	Amy E. Mandragouras	Reg. No. 36,207
Thomas V. Smurzynski	Reg. No. 24,798	Elizabeth A. Hanley	Reg. No. 33,505
Ralph A. Loren	Reg. No. 29,325	Matthew P. Vincent	Reg. No. 36,709
Thomas J. Engellenner	Reg. No. 28,711	Paul Louis Myers	Reg. No. 35,965
William C. Geary III	Reg. No. 31,359	Beth E. Arnold	Reg. No. 35,430
David J. Powsner	Reg. No. 31,868	Anthony A. Laurentano	Reg. No. 38,220
Giulio A. DeConti, Jr.	Reg. No. 31,503	Jane E. Remillard	Reg. No. P38,872
Michael I. Falkoff	Reg. No. 30,833	Jean M. Silveri	Reg. No. P39,030
Ann Lamport Hammitte	Reg. No. 34,858	Mark A. Kurisko	Reg. No. P38,944
		Edward J. Kelly	Reg. No. P38,936

Send Correspondence to:

 Lahive & Cockfield, 60 State Street, Boston, MA 02109

Direct Telephone Calls to: (name and telephone number)

 Guilio A. DeConti, Jr. or Matthew P. Vincent, (617) 227-7400

Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor David H. Beach
Inventor's signature *David H. Beach*   Date 25ᵗʰ May 1995
Residence 19 Woodland Drive, Huntington Bay, NY 11743
Citizenship United Kingdom
Post Office Address (if different)

-4-

Full name of second inventor: Konstantin Galaktionov	
Inventor's signature	Date May 25, 1995
Residence Cold Spring Harbor Laboratory, One Bungtown Road, Cold Spring Harbor, NY 11724	
Citizenship Russia	
Post Office Address (if different)	